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RESEARCH ARTICLE

CHOP/caspase-3 signal pathway involves in mitigative effect of selenium on lead-induced apoptosis via endoplasmic reticulum pathway in chicken testes

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Abstract

Lead (Pb) is an environmental pollutant. Selenium (Se) has alleviative effect on Pb poisoning. However, mitigative effect of Se on Pb-induced apoptosis has not been unclear via endoplasmic reticulum (ER) pathway in chicken testes. The aim of this study was to investigate mitigative effect of Se on apoptosis induced by Pb poisoning via ER pathway in chicken testes. Sixty male chickens (7-day-old) were randomly divided into the control group offered drinking water (DW) and basic diet (BD) (0.49 mg/kg Se), the Se group offered DW and BD containing Na_2SeO_3 (SeBD) (1.00 mg/kg Se), the Pb group offered DW containing $(CH_3OO)_2Pb$ (PbDW) (350.00 mg/L Pb) and BD, and the Pb + Se group offered PbDW and SeBD; and were fed for 90 days. The following contents were performed as follows: histology; antioxidant indexes (reduced glutathione (GSH), malondialdehyde (MDA), glutathione peroxidase (GPx), glutathione *S*-transferase (GST), and superoxide dismutase (SOD)); mRNA expressions of ER-related genes (glucose-related protein 78 (GRP78), protein kinase-like ER kinase (PERK), eukaryotic initiation factor 2α (eIF2α), activating transcription factor 4 (ATF4), and enhancer-binding protein homologous protein (CHOP)); and apoptosis-related genes (cysteine-aspartic protease (caspase)-3 and caspase-12) in chicken testes. The results indicated that Pb poisoning caused histological changes; increased MDA content; decreased the content of GSH and the activities of GPx, GST, and SOD; and upregulated mRNA expressions of the above five ER-related genes and two apoptosis-related genes in the chicken testes. Se alleviated Pb-induced oxidative stress, ER stress, and apoptosis via CHOP/caspase-3 signal pathway in the chicken testes.

Keywords Selenium · Lead · Chicken · Testis · Apoptosis · CHOP/caspase-3 signal pathway

Introduction

Lead (Pb) was widely used in industrial and agricultural productions, which led to environmental pollution. Pb pollution affected reproduction in female wild birds. Gasparik et al. (2012) reported that Pb accumulated in livers, kidneys, pectoral muscles, ovaries, and eggs; and decreased egg weight, fertilization rate, and egg hatching ratio in pheasants. Pb was harmful for hatchability, fertility, and reproductive success of Japanese quail (Butkauskas and Sruoga 2004). Pb poising also damaged chicken testes (Wang et al. 2017b).

Indicators of oxidative stress (such as reduced glutathione (GSH), malondialdehyde (MDA), glutathione peroxidase (GPx), glutathione *S*-transferase (GST), and superoxide dismutase (SOD)) were related to Pb poisoning. GSH, MDA, GPx, GST, and SOD participated in Pb-induced oxidative stress in the proximal tubular cells (Wang et al. 2009) and livers (Rahmanand Sultana 2006) and testes (Mabrouk and Cheikh 2015) of rats.

Endoplasmic reticulum (ER) is an important and multifunctional organelle. Oxidative stress can cause excessive accumulation of unfolded and misfolded proteins in the ER, and induce ER stress (Hanada et al. 2007). Glucose-regulated protein 78 (GRP78), known as master resident ER chaperone, is

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an important regulator of ER function. PRKR-like ER kinase (PERK) is one of major ER membrane transducers. The activation of PERK alleviates ER stress by inhibiting protein transcription, expanding the ER membrane, and elevating chaperone level (Liu et al. 2017a). The activation of PERK leads to eukaryotic translation initiation factor 2α (eIF2α) phosphorylation and induces selectively activating transcription factor 4 (ATF4) (a transcription factor). Then, the activity of ATF4 enhances PERK-enhancer-binding protein homologous protein (CHOP). Cysteine-aspartic protease (caspase)-12 locates in ER and is specifically involved in apoptosis induced by ER stress. Activated caspase-12 causes caspase-3 activation. And activated caspase-3 leads to apoptosis. Corsetti et al. (2017) found that Pb caused ER stress and apoptosis in mouse spleens. Prolong ER stress increased CHOP expression and caused apoptosis in rat hypothalamuses (Yi et al. 2017).

Selenium (Se) has antioxidant function (Yao et al. 2013a, b). Se not only antagonized the accumulation of Pb but also promoted the excretion of Pb in rat kidneys (Flora et al. 1983). Many studies have shown that Se antagonized Pb-induced DNA damage in HepG2 cells (McKelvey et al. 2015) and oxidative stress in human serum (Pawlas et al. 2016). Se alleviated damages in bursa of Fabricius (Jiao et al. 2017), livers (Xu et al. 2016), kidneys (Wang et al. 2018), and testes (Wang et al. 2017b) in Pb-treatment chickens; and nerve damage in hippocampus (Wang et al. 2013) and reproductive damage in ovaries (Shen et al. 2016) in Pb-treatment rats. Jin et al. (2017) found that Se alleviated Pb-induced apoptosis in chicken kidneys. However, the mechanism of Se antagonized Pb-induced apoptosis in chicken testes remains unclear.

At present, whether ER stress involved in mitigative effect of Se on apoptosis induced by Pb in poultry testes has not been reported. Therefore, we designed Pb poisoning-Se antagonism model and researched histology, antioxidant indexes, and mRNA expressions of ER-related genes and apoptosis-related genes to investigate alleviative effect of Se on Pb-induced apoptosis via CHOP/caspase-3 signal pathway in chicken testes.

Materials and methods

Animals

All procedures used in this study were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University. Sixty Hyline male chickens (1-day-old) with similar initial body weights (40.00 ± 0.30 g) were randomly divided into four groups after being fed basic diet (BD) (containing 0.49 mg/kg Se) and drinking water (DW) for 7 days. Each group consisted of three replicates (five chickens per replicate). The control group was fed DW and BD. The Se group was fed BD containing Na_2SeO_3 (SeBD) (1.00 mg/kg Se) and DW. The Pb group was fed BD and DW containing $(CH_3OO)_2Pb$ (PbDW) (350.00 mg/L Pb). The use of Pb dose was conducted in the needs of experiment toxicology (Klaassen and Watkins 2013), according to a median lethal does (LD50) of Pb for chickens (Vengris and Mare 1974). The Pb + Se group was fed SeBD and PbDW. The chickens were housed in the Laboratory Animal Center, College of Veterinary Medicine, Northeast Agricultural University, China, with a controlled environment (natural illumination, room temperature 22 ± 2 °C, and 50 ± 5% relative humidity). Food and water were provided ad libitum throughout the trial period. All treatments lasted for 90 days.

Tissue samples

All chickens were euthanized at the end of treatments. Testes were quickly stripped and washed with deionized water. Each testis was cut into roughly four equal parts. The first part was immediately frozen in liquid nitrogen and stored at − 80 °C until required for relative mRNA expression. The second part was cut into small pieces and put into cold sterile sodium chloride solution for the determination of antioxidant indexes. The third part was fixed in 5% paraformaldehyde solution and stayed at least 24 h for microstructure and for TdT-mediated dUTP nick end labeling (TUNEL) assay. And the final part was fixed in 2.5% glutaraldehyde fixation for ultrastructure.

Microstructure

The testis tissue fixed with paraformaldehyde solution was dehydrated in gradient alcohol (50, 75, 85, 95, 95, 100, and 100%), embedded in paraffin, and sectioned at 4 μm. The sections were stained with hematoxylin and eosin and were observed using light microscopy.

Ultrastructure

The samples fixed in glutaraldehyde were performed ultrathin section. The sections were stained with uranyl acetate and lead citrate and were observed using transmission electron microscope (TEM, model JEM-1200EX, JEOL JEM, Japan).

Indices related to oxidative stress

Small pieces of testes were taken from cold sterile sodium chloride solution and were ground in a homogenization buffer (0.05 M Tris-HCl pH 7.9, 25% glycerol, 0.10 mM EDTA, and 0.32 M $(NH_4)_2SO_4$). A homogenizer was used to homogenize the lysates on ice. The solution was centrifuged at 16,000 g at 4 °C for 5 min. The supernatant was used for determining GSH and MDA contents and GPx, GST, and SOD activities in the testes of chickens using kits produced by Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's instructions.

TUNEL assay

In Situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) was used to detect apoptosis according to the manufacturer's instructions. The tissue sections embedded in paraffin were treated with proteinase K. Endogenous peroxidase activity was blocked with hydrogen peroxide. The sections were incubated with a terminal TdT/nucleotide mixture at 37 °C for 1 h and were rinsed with phosphate-buffered saline. Nuclear labeling was performed with horseradish peroxidase and diaminobenzidine. The sections were counterstained with hematoxylin. More than 1000 cells coming from random 50 fields per slide were counted at 400× magnification, and the number of positively stained nuclei was recorded. The extent of apoptosis was presented as apoptosis index (AI). AI was a percentage of apoptotic cells in total cells.

Relative mRNA expression

The special primer sequences of GRP78, PERK, ATF4, eIF2α, CHOP, caspase-12, caspase-3, and GADPH published in GenBank were listed in Table 1. GADPH was used as an internal reference gene. The primers were synthesized by Invitrogen Biotechnology Co., Ltd. in Shanghai, China.

Total RNA was isolated from testis samples using TRIzol reagent following the method provided by the manufacturer (Invitrogen, China). Spectrophotometer (Healthcare Bio-Sciences AB, Sweden) was used to determine RNA purity. OD260/OD280 was between 1.8 and 2.1. Complementary DNA (cDNA) was synthesized using PrimeScript™ RT reagent Kit (TaKaRa, Japan) in a volume of 60 μL (containing 5 μg of the total RNA) according to the manufacturer's instructions. Synthesized cDNA was diluted fivefold with sterile water and was stored at − 20 °C until the next step.

Quantitative real-time PCR was performed using LightCycler® 96 (Roche, Life Science) with the SYBR® PrimeScript™ RT-PCR Kit (Roche, Switzerland) according to the manufacturer's instructions. Reactions were performed in a 10-μL reaction mixture containing 5 μL of the 2× SYBR Green I PCR Master Mix, 1 μL cDNA, 0.3 μL of each primer (10 μM), and 3.4 μL of PCR-grade water. PCR procedure consisted of 95 °C for 10 min, followed by 40 cycles of denaturing-annealing/elongating (95 °C for 15 s and 60 °C for 1 min), and melting curve analysis (95 °C for 15 s and 60 °C for 20 s). The melting curve analysis showed only one peak for each PCR product. There were three duplications for each sample. Relative mRNA expressions were calculated according to the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

All data were presented as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was performed using SPSS (version 19, SPSS Inc., Chicago, IL, USA). Kruskal-Wallis ANOVA test and Mann-Whitney *U* test were used to verify the comparison among groups. The results were considered to be significant when $P < 0.05$.

Results

Testicular histology

Histological change was a direct indication of testis damage. Therefore, we researched microstructure (Fig. 1a1–d1), ultrastructure (Fig. 1a2–d2), and TUNEL assay (Fig. 1a3–d3) in the chicken testes. Microstructure results of chicken testes were as follows: in the control (Fig. 1a1) and Se (Fig. 1b1) groups, seminiferous tubules developed normally. Spermatogenic cells displayed clear profile, complete structure, and orderly arrangement. In the Pb group (Fig. 1c1), blood-testis barrier was seriously damaged. Seminiferous tubules deformed. Some vacuoles appeared in the genital epithelium. The nuclei of spermatogenic cells shrank. Some spermatogenic cells fell off into lumens. Compared with the control and Se groups, genital epithelium thinned. Spermatogenic cells arranged irregularly. And the number of spermatogenic cells decreased obviously. In the Pb + Se group (Fig. 1d1), spermatogenic tubes deformed. Spermatogenic cells arranged irregularly. A few spermatogenic cells fell off into lumens. And the number of spermatogoniums was less than that in the control group, but more than that in the Pb group.

Ultrastructure of chicken testes was shown in Fig. 1a2–d2. The control (Fig. 1a2) and Se (Fig. 1b2) groups showed evenly distributed chromatin, clear nuclei, obvious nucleoli, and intact ER. The Pb group (Fig. 1c2) showed hyperchromatic nuclei, incomplete nuclear membranes, swollen ER, unclear profile of ER, and vacuoles in the ER. In the Pb + Se group (Fig. 1d2), the changes of nuclei and ER were less than those in the Pb group.

The TUNEL assay results of chicken testes were shown in Fig. 1a3–d3. In the control (Fig. 1a3) and Se (Fig. 1b3) groups, a large number of nuclei were blue and a few nuclei were yellow brown. In the Pb group (Fig. 1c3), lots of yellow brown nuclei appeared. The number of yellow brown nuclei in the Pb + Se group (Fig. 1d3) was less than that in the Pb group, but more than that in the control and Se groups. In addition, we analyzed AI. As shown in Fig. 2, there were significant differences ($P < 0.05$) in AI among different groups except that there were no significant differences ($P > 0.05$) in AI between the control group and the Se group. AI (30.48%) of the Pb group was the highest, followed by 18.83% of the Pb + Se group, 4.06% of the control group, and 3.86% of the Se group. AI of the Pb group was 7.51 times as much as that of the control group.

Table 1 Special primers of genes used for real-time PCR

Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
GRP78	GAATCGGCTAACACCAGAGGA	CGCATAGCTCTCCAGCTCATT
PERK	TCATCCAGCCTCAGTAAACC	ACAACATCCTCGCCCAGT
eIF2α	CAGGGGCACCCAACCTACAA	CGGGCACAAATACTTTCATCATCT
ATF4	GAATCGGCTAACACCAGAGGA	CGCATAGCTCTCCAGCTCATT
CHOP	AGCCACATCACCACTTCCC	CACGCTTCCGCTTTGTCCT
Caspase-12	AATAGTGGGCATCTGGGTCA	CGGTGTGATTTAGACCCGTAAGAC
Caspase-3	CATCTGCATCCGTGCCTGA	CTCTCGGCTGTGGTGGTGAA
GADPH	AGAACATCATCCCAGCGT	AGCCTTCACTACCCTCTTG

Antioxidant index

Antioxidant indexes of chicken testes were shown in Fig. 3. There were no significant differences ($P > 0.05$) in the contents of GSH and MDA and the activities of GST and SOD between the control group and the Se group. However, GPx activity in the Se group was significantly higher ($P < 0.05$) than that in the control group. MDA content in the Pb group was significantly higher ($P < 0.05$) than that in the other three groups. MDA content in the Pb + Se group was significantly higher ($P < 0.05$) than that in the control and Se groups. Change tendency of GSH content and GPx, GST, and SOD activities were opposite to that of MDA content.

Relative mRNA expression

As shown in Fig. 4, mRNA expressions of GRP78, PERK, eIF2α, ATF4, CHOP, caspase-3, and caspase-12 in the Pb group increased significantly ($P < 0.05$) compared with those in the control, Se, and Pb + Se groups. Moreover, mRNA expression of GRP78 in the Pb group was 12.53 times that of GRP78 in the control group. Relative mRNA expressions of all detected genes in the Pb + Se group were significantly higher ($P < 0.05$) than those in the control and Se groups. There were no significant differences ($P > 0.05$) in relative mRNA expressions of all detected genes between the control group and the Se group.

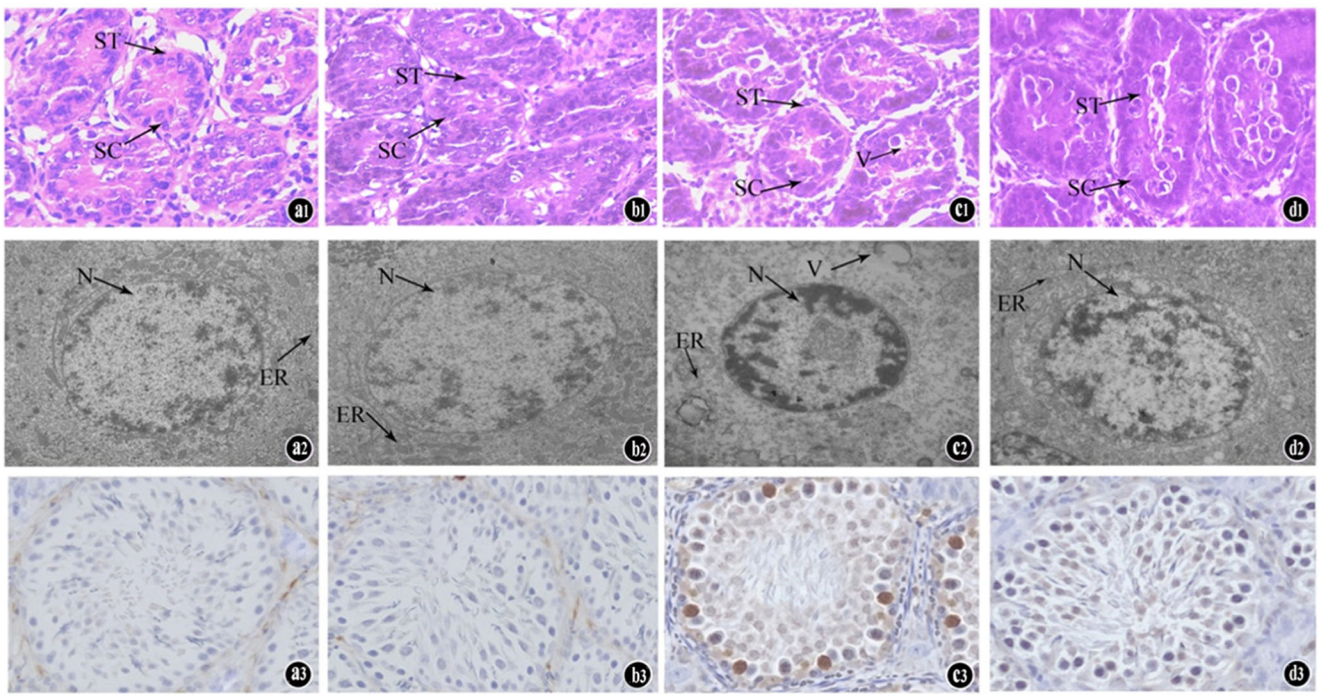

Fig. 1 Effects of Pb, Se, and their co-treatment on histology of chicken testes after 90 days. The control group (**a1** × 400; **a2** × 12,000; **a3** × 1000); the Se group (**b1** × 400; **b2** × 12,000; **b3** × 1000); the Pb group (**c1** × 400; **c2** × 12,000; **c3** × 1000); the Pb + Se group (**d1** × 400; **d2** × 12,000; **d3** × 1000). ST seminiferous tubule, SC spermatogenic cell, V vacuole, N nucleus, ER endoplasmic reticulum

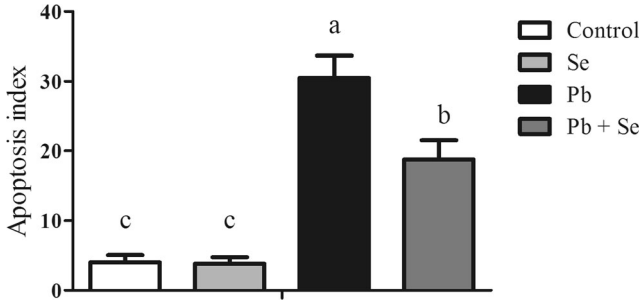

Fig. 2 Effects of Pb, Se, and their co-treatment on apoptosis index of chicken testes after 90 days. *Data* represent mean ± SD ($n = 5$). Statistically significant differences: *data with different lowercase letters* among different groups are significantly different ($P < 0.05$)

Discussion

Our previous study found that excess Pb led to Pb accumulation and inflammatory injury in chicken testes and demonstrated that chicken testis was a target organ of Pb (Wang et al. 2017b). In our study, we also found that Pb damaged testes and induced apoptosis in the chicken testes.

Many researchers found that Pb treatment caused oxidative stress. GSH, MDA, GPx, GST, and SOD are oxidative stress markers. Jiao et al. (2017) reported that Pb poisoning increased MDA content; decreased GSH content; and decreased GPx, GST, and SOD activities in chicken bursa of Fabricius. Excess Pb increased MDA content and decreased GSH content and GST activity in *Cyprinus carpio* livers (Özkan-Yilmaz et al. 2014). In our study, we investigated GSH and MDA contents as well as GPx, GST, and SOD activities in the chicken testes. Our results were consistent with the previous studies. The results showed that the Pb treatment increased MDA content; decreased GSH content; decreased GPx, GST, and SOD activities; and induced oxidative stress in the chicken testes.

Pb-induced oxidative stress caused ER stress in rat livers (Liu et al. 2013) and in mouse spleens (Corsetti et al. 2017) and apoptosis in mouse spleens (Corsetti et al. 2017). The accumulating unfolded and misfolded proteins caused dissociation of GRP78, PERK, and caspase-12. GRP78, a specific marker of ER stress, is only expressed in ER. Pb caused ER stress and GRP78 protein expression in *Ctenopharyngodon idellus* kidney cells (Zhong et al. 2017). Se deficiency led to oxidative stress and ER stress and increased GRP78 protein expression in chicken livers (Yao et al. 2015). In our study, Pb treatment increased dramatically GRP78 mRNA expression. GRP78 mRNA expression in the Pb group was 12.53 times as much as that in the control group. The results indicated that Pb treatment caused ER stress in the chicken testes. Dissociated PERK, a major transducer of ER stress, is activated. And activation of PERK leads to the phosphorylation of eIF2α and then sensitizes transcription factor ATF4. Sustained overexpression of ATF4 upregulates CHOP expression. CHOP is a critical mediator of ER stress-induced apoptosis. CHOP expresses at low level under normal physiological conditions. However, CHOP expresses at high level during prolonged ER stress and then induces apoptosis. After ER stress, caspase-12 dissociates and is activated and further activates caspase-3. Then, caspase-3 executes apoptosis. In our study, we found that Pb treatment upregulated PERK, eIF2α, ATF4, CHOP, caspase-12, and caspase-3 mRNA expressions in the chicken testes.

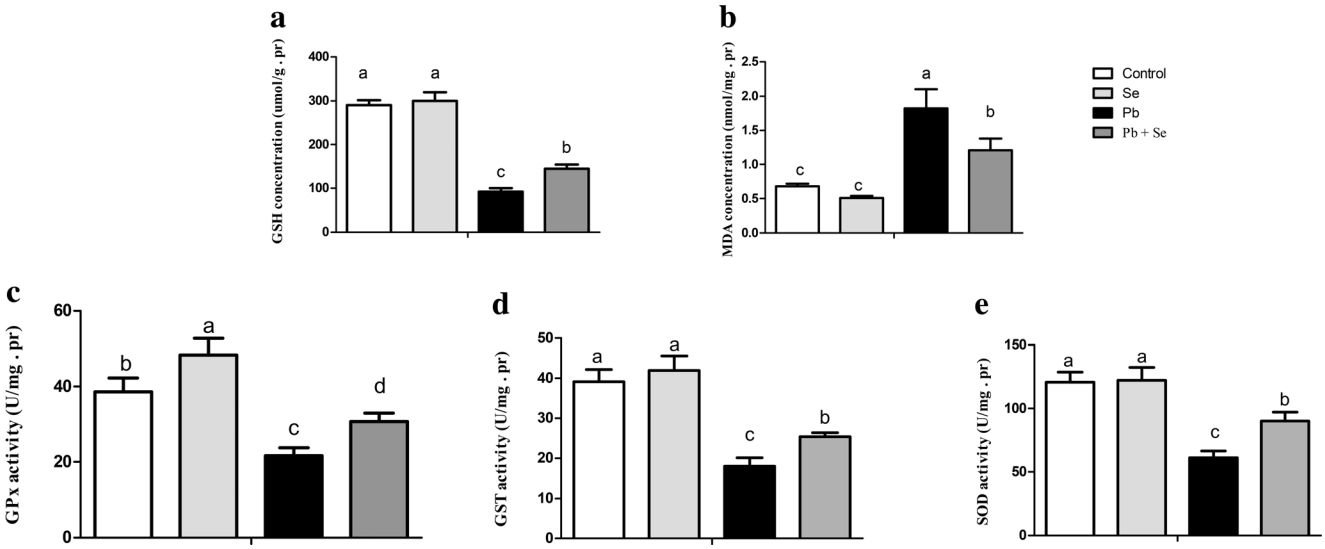

Fig. 3 Effects of Pb, Se, and their co-treatment on GSH (**a**) and MDA (**b**) contents; and GPx (**c**), GST (**d**), and SOD (**e**) activities in chicken testes after 90 days. Fifteen chickens consisted of three replicate pens, with each pen containing five chickens. *Data* represent mean ± SD ($n = 5$). Statistically significant differences: *data with different lowercase letters* in the same index among different groups are significantly different ($P < 0.05$)

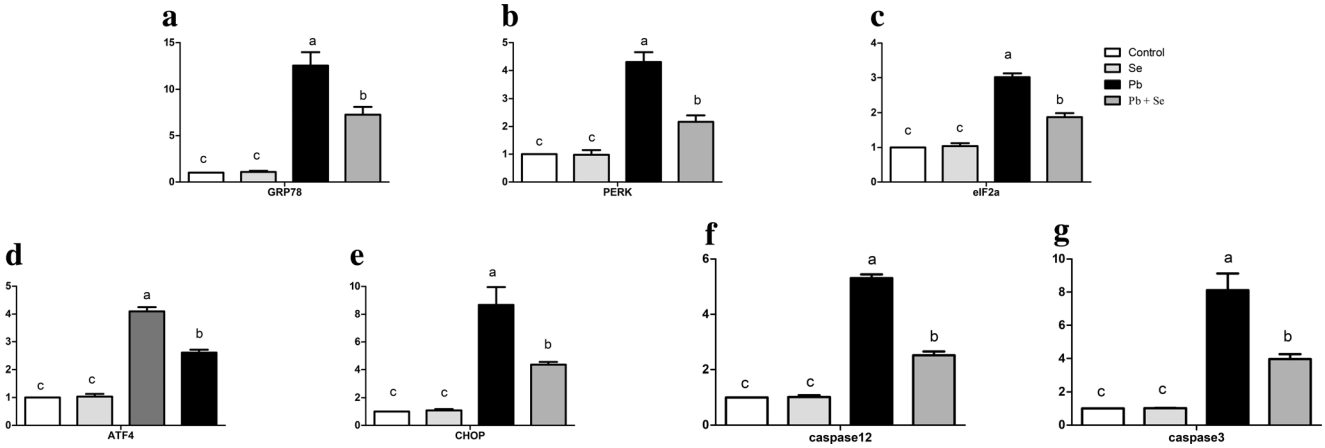

Fig. 4 Effects of Pb, Se, and their co-treatment on relative mRNA expressions of GRP78 (**a**), PERK (**b**), eIF2α (**c**), ATF4 (**d**), CHOP (**e**), caspase-3 (**f**), and caspase-12 (**g**) in chicken testes after 90 days. Fifteen chickens consisted of three replicate pens, with each pen containing five chickens. *Data* represent mean ± SD ($n = 5$). Statistically significant differences: *data with different lowercase letters* in the same gene among different groups are significantly different ($P < 0.05$)

Our results indicated that Pb induced ER stress and apoptosis via CHOP/caspase-3 signal pathway in the chicken testes. Our researches were consistent with others. Pb induced PERK and eIF2α protein expressions, ER stress, and apoptosis in rat brains (Liu et al. 2014). Pb treatment increased GRP78, CHOP, and caspase-12 protein expressions and induced ER stress and apoptosis in primary cultures of rat proximal tubular cells (Wang et al. 2017a). GRP78, PERK, ATF4, CHOP, and caspase-3 expressions increased; and ER stress and apoptosis occurred in Pb-exposed rat livers (Liu et al. 2017b).

Se can alleviate Pb accumulation induced by excess Pb in chicken testes (Wang et al. 2017b). Some studies indicated that Se alleviated oxidative stress induced by Pb. Se alleviated Pb-induced oxidative stress by decreasing MDA content and increasing GSH content and GST activity in *Cyprinus carpio* livers (Özkan-Yilmaz et al. 2014), by increasing SOD and GPx activities and decreasing MDA content in chicken kidneys (Jin et al. 2017). We got the similar the results of the previous researches. We found that Se alleviated the increase of MDA content; the decrease of GSH content; the decrease of GPx, GST, and SOD activities; and oxidative stress in the Pb-caused chicken testes. Se is the active center of GPx and can increase GPx activity. In our study, we found that Se increased GPx activity. Our result further demonstrated that Se alleviated Pb-induced oxidative stress in the chicken testes. Moreover, Se was reported to have protective effects on apoptosis. Se alleviated the increase of caspase-3 mRNA and protein expressions and apoptosis in the Pb-induced chicken kidneys (Jin et al. 2017). Se alleviated the increase of GRP78, ATF4, caspase-12, and caspase-3 expressions; ER stress; and apoptosis in Pb-induced chicken kidneys (Wang et al. 2018). We also determined mRNA expressions of GRP78, ATF4, caspase-12, and caspase-3 in chicken testes. We found that Se alleviated Pb-induced increase of GRP78, ATF4, caspase-12, and caspase-3 mRNA expressions; ER stress; and apoptosis in the chicken testes. Moreover, we measured PERK, eIF2α, and CHOP; and found that Se alleviated Pb-induced increase of PERK, eIF2α, and CHOP mRNA expression in the chicken testes. Our above results indicated that Pb-induced oxidative stress, ER stress, and apoptosis were alleviated by Se supplement via CHOP/caspase-3 signal pathway (Fig. 5) in the chicken testes.

Conclusion

Pb poisoning changed histology structure; increased MDA content; decreased GSH content; decreased GPx, GST, and SOD activities; and increased mRNA expressions of

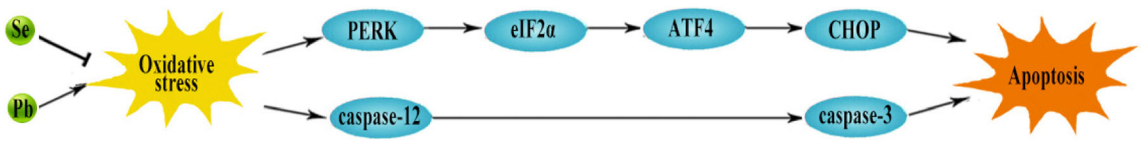

Fig. 5 CHOP/caspase-3 signaling pathway of Se-alleviated apoptosis induced by Pb in the chicken testes

GRP78, PERK, eIF2α, ATF4, CHOP, caspase-12, and caspase-3 in the chicken testes. Se alleviated the above changes caused by Pb. Excess Pb induced oxidative stress, ER stress, and apoptosis via CHOP/caspase-3 signal pathway in the chicken testes.

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Compliance with ethical standards

All procedures used in this experiment were approved by the Northeast Agricultural University's Institutional Animal Care and Use Committee under the approved protocol number SRM-06.

Conflict of interest The authors declare that they have no conflicts of interest.

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