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RESEARCH ARTICLE

Alleviative effect of selenium on inflammatory damage caused by lead via inhibiting inflammatory factors and heat shock proteins in chicken testes

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Abstract The aim of this study was to investigate ameliorative effect of selenium (Se) on lead (Pb)-induced inflammatory damage in chicken testes. One hundred eighty 7-day-old male chickens were randomly assigned into the control group, the Se group, the Pb group, and the Pb/Se group. Lead acetate was added in drinking water (350 mg/L Pb). Sodium selenite was added in the standard commercial diet (1 mg/kg Se). On the 30th, 60th, and 90th days of the experiment, 15 chickens of each group were euthanized. Inductively coupled plasma mass spectrometry, hematoxylin and eosin staining, real-time quantitative PCR, and Western blot were used. The results indicated that excess Pb increased nitric oxide content; inducible nitric oxide synthase (iNOS) activity; nuclear factor-kappa B (NF-κB), tumor necrosis factor-α, cyclooxygenase-2, prostaglandin E synthases, and iNOS mRNA levels in a time-dependent manner; NF-κB, iNOS, heat shock protein (HSP) 60, HSP70, and HSP90 protein levels; and Pb concentration. Excess Pb decreased Se concentration and induced histological changes. Se-alleviated Pb caused all of the above changes. Se improved Pb-caused inflammatory damage by decreasing the expression of inflammatory factors and heat shock proteins in the chicken testes. Our results provided theoretical basis of an alleviative effect of Se on Pb-induced bird testis damage.

Keywords Lead acetate · Sodium selenite · Bird testis · NF-κB · Heat shock protein · Inflammatory damage

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Introduction

Lead (Pb) is an environmental toxicant that is used in industry, such as battery, pigment, alloy, and gasoline additive (Mao et al. 2008). It can pollute the environment and even injure the health of human beings (Assi et al. 2016), mammals (El-Ashmawy et al. 2006; Ashry et al. 2010; El-Sayed and El-Neweshy 2010), and birds (Osickova et al. 2012). Chronic Pb exposure damaged sperm structure of men who worked at a lead acid battery factory (Naha et al. 2005). Abdel-Moneim (2014) found that human activities, such as agriculture, urban, and industrial developments, caused high Pb concentration and the damage of tilapia livers in Lake Al-Hassa, Saudi Arabia. Pb pollution caused inflammatory leukocyte infiltration in the livers and kidneys of mallards and coots near a fishpond in southern Poland (Binkowski et al. 2013). Wild birds had high blood Pb concentration in Pb-polluted mining regions at Mexico (Chapa-Vargas et al. 2010). Pb-induced cytotoxicity in primary rat proximal tubular cells (Liu et al. 2014) changed histological structure in rat livers (Mabrouk et al. 2016) and damaged rat renal tissue (Liu et al. 2010). The testis is one of the target organs of Pb toxicity. Pb damaged the testes of frogs (Wang and Jia 2009) and rats (El-Neweshy and El-Sayed 2011).

Heavy metal poisoning can cause an inflammatory response. Nuclear factor-kappa B (NF-κB), a crucial inflammatory factor, is closely related to tumor necrosis factor-α

(TNF-α), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) (Hseu et al. 2006). Nitric oxide (NO) derives from iNOS and plays a significant role in the pathogenesis of inflammation (Sharma et al. 2007). A study showed that NF-κB influenced the course of mucosal inflammation strongly in patients with inflammatory bowel disease (Atreya et al. 2008). The increase of NF-κB level aggravated the severity of pancreatitis (Huang et al. 2013). Molybdenum and cadmium (Cd) upregulated messenger RNA (mRNA) expression of NF-κB, TNF-α, and COX-2 in the livers (Cao et al. 2016a) and kidneys (Cao et al. 2016b) of ducks. Our previous studies demonstrated that excess manganese (Mn) caused mRNA expression of inflammatory factors, inflammation response, and inflammatory injury in chicken testes (Du et al. 2015); Pb poisoning resulted in mRNA expression of inflammatory factors and inflammation response in chicken livers (Wang et al. 2015).

Heat shock proteins (HSPs) are primary molecular chaperones that have important functions which translocate proteins and assist folding/unfolding and assembly/disassembly of protein complexes (Bernabò et al. 2011). HSP70 and HSP90 have protective effects against stress-induced cellular damage (Ivanina et al. 2008). Some studies showed that stressors could increase the levels of HSP60, HSP70, and HSP90. For example, cold stress induced protein expression of HSP60, HSP70, and HSP90 in chicken immune organs (Zhao et al. 2014a). Harmful toxicants increased HSP70 mRNA level in the livers, brains, and kidneys of common carp (Xing et al. 2013). Arsenic (As) increased HSP70 and HSP90 mRNA levels in chicken immune organs (Guo et al. 2016).

Selenium (Se) is an essential trace element (Yao et al. 2013a, b). It associates with inflammatory cytokines (Jiang et al. 2015; Liu et al. 2015a) and HSPs (Yang et al. 2015). Se can protect against Pb-caused changes of ion profiles in chicken muscle tissue (Jin et al. 2016) and selenoprotein mRNA expression in chicken cartilage tissue (Gao et al. 2016).

However, an alleviative effect of Se on Pb-induced toxicity is still unclear in chicken testes. Therefore, we wanted to research and clarify the mechanism of Pb-induced chicken testis damage and an alleviative effect of Se on Pb. Histopathology and measurements of Pb and Se concentrations were performed; and NO content, iNOS activity, inflammatory factor mRNA and protein expression, and heat shock protein expression were detected.

Materials and methods

Animal model and tissue samples

All procedures used in this study were approved by the Institutional Animal Care and Use Committee of the Northeast Agricultural University under the approved protocol number SRM-06. One hundred eighty 1-day-old healthy Hyline male chickens were raised on a standard commercial diet (containing 0.49 mg/kg Se) and drinking water for 1 week. Subsequently, the chickens were randomly assigned into four groups (45 chickens per group), including the control group, the Se group, the Pb group, and the Pb/Se group. The feeding programs of Pb and Se were as follows: lead acetate ($(CH_3OO)_2Pb$) was added in drinking water (containing 350 mg/L Pb), based on the median lethal dose (LD_{50}) of Pb for chickens (Vengris and Mare 1974) and the need for chicken experiments in toxicology (Klaassen and Watkins 2013). Sodium selenite (Na_2SeO_3) was added in the standard commercial diet (containing 1 mg/kg Se). Lead acetate and sodium selenite were bought from Tianjin Zhiyuan Chemical Reagent Co., Ltd., China. The chickens were housed in the Laboratory Animal Center of the Northeast Agricultural University (Harbin, China). The chickens were given ad libitum access to feed and water in accordance with chicken feeding operation procedures.

The chickens were randomly selected (n = 15/group) and euthanized on the 30th, 60th, and 90th days of the experiment. Next, testes were quickly removed and flushed with ice-cold, sterile deionized water. One portion of the samples was immediately frozen in liquid nitrogen and stored at −80 °C to perform Pb and Se concentration analysis, real-time quantitative PCR, and Western blot. One portion of the samples was fixed in 5% paraformaldehyde solution for histopathology. The remaining portion of the samples was homogenized to determine NO content and iNOS activity.

Pb and Se concentrations

On the 90th day, each sample (1 g) was digested in a microwave digestion system with 5 mL of HNO_3 (65%) and 2 mL of H_2O_2 (30%) and diluted to 10 mL with deionized water. The blank group digest was performed by the same method. The solutions obtained were clear. The microwave system was performed under the following conditions: 3 min for 1800 W at 100 °C, 10 min for 1800 W at 150 °C, and 45 min for 1800 W at 180 °C. The digested samples were filled with ultrapure water and then analyzed by inductively coupled plasma mass spectrometry (ICP-MS; Thermo iCAPQ, USA).

Histopathology

Dehydrating, clearing, embedding, baking, and hematoxylin and eosin (H & E) staining were performed following the method described in our previous paper (Du et al. 2015). The histological changes were observed using a light microscope (Eclipse 80i, Nikon, Tokyo, Japan).

NF-κB, TNF-α, COX-2, PTGEs, and iNOS mRNA expression

Primer synthesis

The primer sequences of inflammatory factors NF-κB (NM_205134), TNF-α (NM_204267), COX-2 (NM_001167718), prostaglandin E synthases (PTGEs, NM_001194983), iNOS (NM_204961), HSP60 (NM_001012916.1), HSP70 (NM_001006685.1), and HSP90 (NM_001109785.1) published in GenBank were synthesized by the Invitrogen Biotechnology Co., Ltd. (Shanghai, China). β-actin (NM_205518) was used as an internal reference gene.

Total RNA extraction and reverse transcription

Total RNA was extracted from the chicken testis samples using TRIzol reagent (TaKaRa, Japan) following the manufacturer's protocol. RNA concentration and purity were examined at 260:280 nm ratio using GeneQuant 1300/100 spectrophotometer (GE Healthcare Bio-Sciences AB, Sweden). Complementary DNA (cDNA) was synthesized. The reverse transcriptase (RT) reaction (60 μL) (HaiGene, Harbin, China) composed of 6 μL of total RNA, 3 μL of golden M-MLV III RT, 6 μL of 10× RT buffer, 3 μL of dNTP mixture (10 mM each), 3 μL of 20× Oligo dT (25), 1.5 μL of RNase inhibitor, and 37.5 μL of RNase-free H_2O. The reaction conditions were at 30 °C for 15 min, at 55 °C for 50 min, and at 80 °C for 10 min. The synthesized cDNA was diluted five times with sterile water and then stored at −20 °C for real-time quantitative PCR.

Real-time quantitative PCR

Real-time quantitative PCR was analyzed using LightCycler® 96 (Roche, Switzerland). The 10 μL reaction mixture contained 5 μL of 2× SYBR Green PCR Master Mix, 0.3 μL each of forward and reverse primers, 3.4 μL of sterile distilled water, and 1 μL of template cDNA. The reaction conditions of the mixture were as follows: 52 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min, 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 expression of mRNA was calculated using Pfaffl method (Pfaffl 2001).

NO content and iNOS activity

The measurements of NO content and iNOS activity were performed using NO and iNOS detection kits according to the instruction of the reagent company (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Western blot

The proteins of NF-κB, iNOS, HSP60, HSP70, and HSP90 were extracted on the 90th day in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The concentration of SDS-polyacrylamide gel was 15 (for NF-κB), 10 (for iNOS), and 12% (for HSP60, HSP70, and HSP90), respectively. The proteins were transferred onto nitrocellulose membranes using a tank at 200 mA for 2 h. The membranes were blocked using 5% skim milk overnight and incubated with diluted first antibody rabbit against NF-κB (1:100, Abcam, USA), antibody rabbit against iNOS (1:200, Abcam, USA), and chicken antibody HSP60 (1:1400), HSP70 (1:500), and HSP90 (1:500). The polyclonal antibody of HSP60, HSP70, and HSP90 were provided by Dr. Xu, College of Veterinary Medicine, Northeast Agricultural University. The membranes were incubated with NF-κB (1:1000), iNOS (1:1000), and HSP (1:1500) horseradish peroxidase (HPR)-conjugated secondary antibody against rabbit IgG (Santa Cruz, CA, USA). The membranes were incubated with the monoclonal β-actin antibody (1:1000) and HPR-conjugated goat antimouse IgG (1:1000). The signals were detected using X-ray films (TransGen Biotech Co., Beijing, China). The optical density of each band was measured using Image VCD gel imaging system (Beijing Sage Creation Science And Technology Co., Ltd., Beijing, China).

Statistical analysis

All the experimental data were analyzed by one-way and two-way analysis of variance (ANOVA) using SPSS for Windows (version 17; SPSS Inc., Chicago, IL, USA). The nonparametric Kruskal-Wallis ANOVA and Mann-Whitney *U* tests were used for verifying statistical comparisons for groups. The values were expressed as the mean ± standard deviation.

Results

Pb and Se concentrations

As shown in Fig. 1, Pb concentration (Fig. 1a) in the Pb group was significantly increased ($P < 0.05$) compared with that all the other groups. Pb concentration was significantly increased ($P < 0.05$) in the Pb/Se group compared with that in the control and Se groups. Se concentration (Fig. 1b) in the Se group was significantly increased ($P < 0.05$) compared with that in all the other groups. Se concentration was significantly decreased

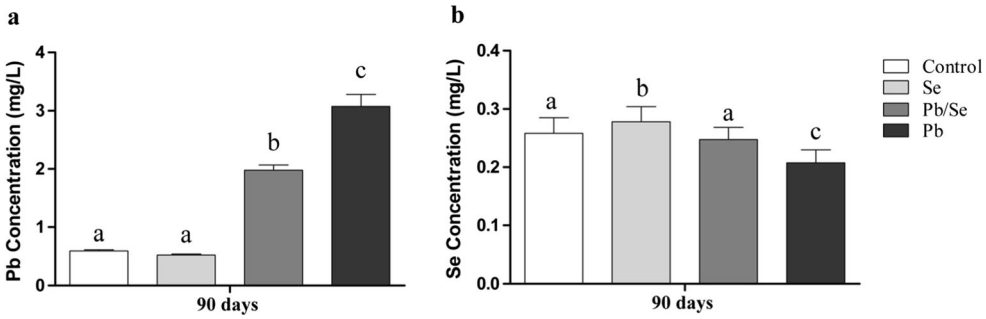

Fig. 1 Pb and Se concentrations on the 90th day in chicken testes. **a** Pb concentration. **b** Se concentration. *Bars* represent mean ± SD. *Bars with different lowercase letters* are significantly different ($P < 0.05$)

($P < 0.05$) in the Pb group compared with that in the control and Pb/Se groups.

Histopathology

Testis histological study was used to determine protective effect of Se on Pb poisoning (Fig. 2). In the control group on the 90th day (Fig. 2a), testis seminiferous tubules showed neatly arranged rows and intact structure. In the Pb group on the 30th day (Fig. 2b), the width of seminiferous tubule interstitial space increased compared with the control group; seminiferous tubule became shrunken and distorted. In the Pb group on the 60th day (Fig. 2c), seminiferous tubules became distorted, germ cells were scattered in the lumen, and germ cells showed irregular shape. In the Pb group on the 90th day (Fig. 2d), seminiferous tubules became severely distorted, germ cells showed severely irregular shape, and some germ cells fell off into the lumen. Moreover, inflammatory cell infiltration increased with the increase of Pb exposure time (Fig. 2b–d). On the 90th day, seminiferous tubules became distorted in the Pb/Se group (Fig. 2e); the extent of seminiferous tubule deformation in the Pb/Se group was lower than that in the Pb group.

NO content, iNOS activity, and iNOS mRNA and protein expression

In the present study, all determined factors (NO content (Fig. 3a), iNOS activity (Fig. 3b), and iNOS mRNA (Fig. 3c) and protein (Fig. 3d) expression) in the Pb group were significantly higher ($P < 0.05$) than those in all the other groups at each time point. NO content, iNOS mRNA, and protein expression in the Pb/Se group were significantly increased ($P < 0.05$) compared with those in the control and Se groups at each time point. The activity of iNOS in the Pb/Se group was significantly increased ($P < 0.05$) compared with that in the control and Se groups except on the 30th day. In the Pb group, NO content, iNOS activity, and mRNA expression displayed significant increase ($P < 0.05$) with the increase of Pb treatment time.

NF-κB, TNF-α, COX-2, and PTGE mRNA and NF-κB protein expression

Real-time PCR results for NF-κB (Fig. 4a), TNF-α (Fig. 4c), COX-2 (Fig. 4d), and PTGEs (Fig. 4e); and Western blot result for NF-κB (Fig. 4b) were given in Fig. 4. All

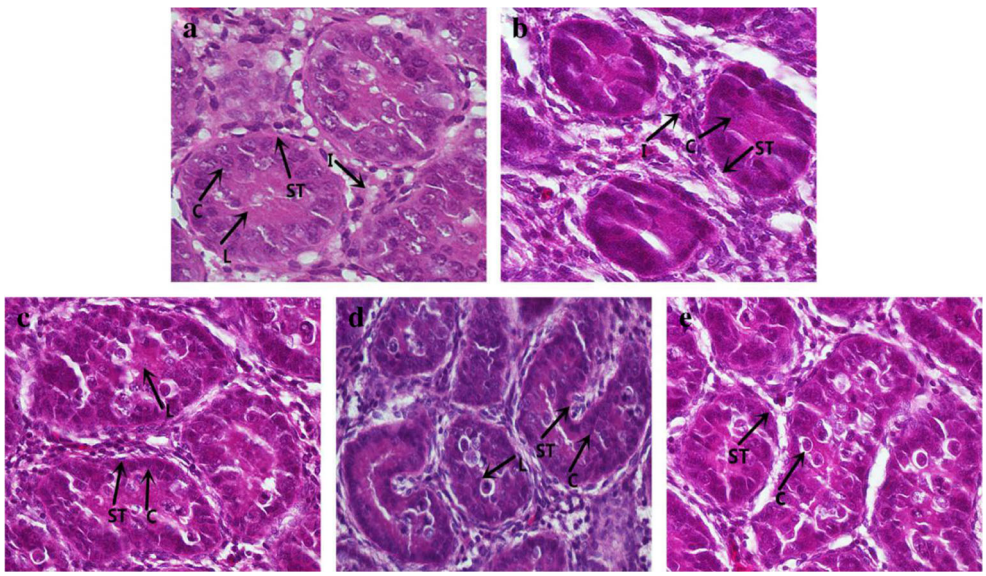

Fig. 2 Histology (hematoxylin and eosin staining, ×400) of chicken testes. **a** The control group on the 90th day. **b** The Pb group on the 30th day. **c** The Pb group on the 60th day. **d** The Pb group on the 90th day. **e** The Pb/Se group on the 90th day. *ST* seminiferous tubules, *I* interstitium, *C* germ cell, *L* lumen

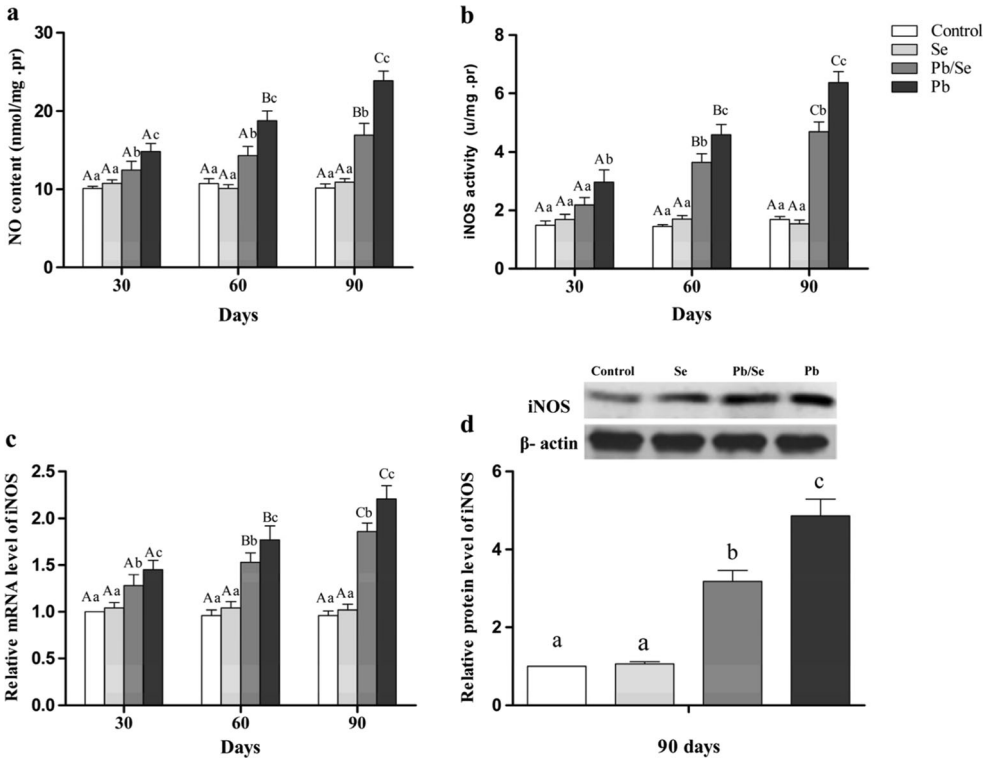

Fig. 3 NO content, iNOS activity, iNOS mRNA, and protein levels in chicken testes. **a** NO content. **b** iNOS activity. **c** iNOS mRNA level. **d** iNOS protein level. *Bars* represent mean ± SD. *Bars with different uppercase letters* are significantly different in the same group at different time points ($P < 0.05$). *Bars with different lowercase letters* are significantly different in different groups at the same time point ($P < 0.05$)

determined inflammatory factors in the Pb group increased significantly ($P < 0.05$) compared with those in all the other groups at each time point. NF-κB mRNA and protein expression and TNF-α mRNA expression in the Pb/Se group were

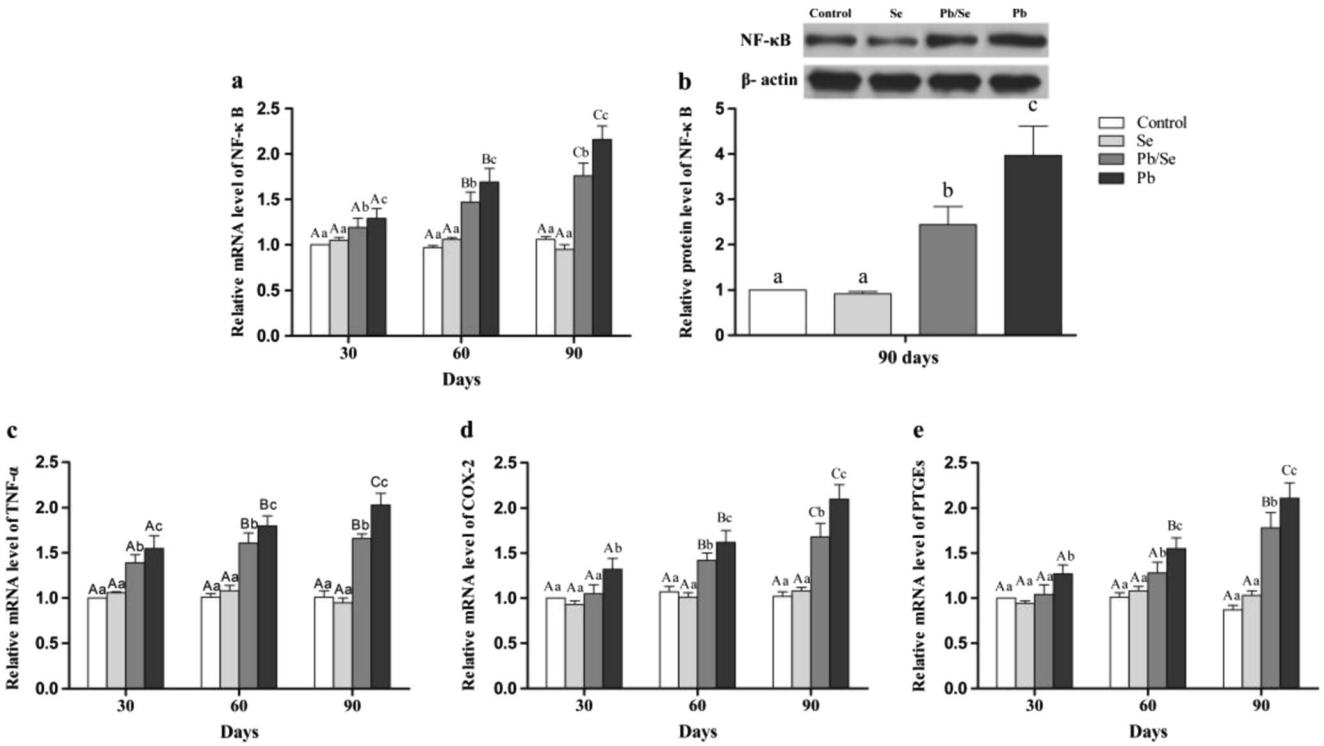

Fig. 4 NF-κB, TNF-α, COX-2, PTGE mRNA, and NF-κB protein levels in chicken testes. **a** NF-κB mRNA level. **b** NF-κB protein level. **c** TNF-α mRNA level. **d** COX-2 mRNA level. **e** PTGEs mRNA level. *Bars* represent mean ± SD. *Bars with different uppercase letters* are significantly different in the same group at different time points ($P < 0.05$). *Bars with different lowercase letters* are significantly different in different groups at the same time point ($P < 0.05$)

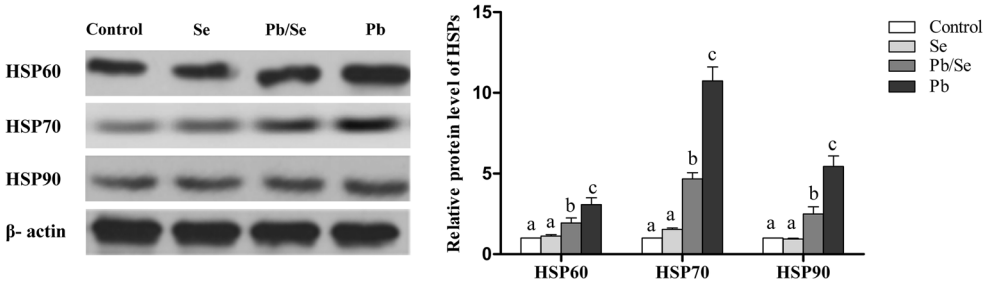

Fig. 5 HSP60, HSP70, and HSP90 protein levels on the 90th day in chicken testes. *Bars* represent mean ± SD. *Bars with different lowercase letters* are significantly different in different groups at the same time point ($P < 0.05$)

significantly higher ($P < 0.05$) than those in the control and Se groups at each time point. COX-2 and PTGE mRNA expression in the Pb/Se group increased significantly ($P < 0.05$) compared with those in the control and Se groups on the 60th and 90th days. In the Pb group, NF-κB, TNF-α, COX-2, and PTGE mRNA expression increased significantly ($P < 0.05$) with the increase of Pb treatment time.

HSP60, HSP70, and HSP90 protein expression

Relative protein expression of HSP60, HSP70, and HSP90 on the 90th day is shown in Fig. 5. All the three HSPs of the Pb group were significantly higher ($P < 0.05$) than those of the other groups. All the three HSPs of the Pb/Se group were significantly higher ($P < 0.05$) than those of the control and Se groups.

Discussion

Excess heavy metals can damage testicular tissue. Cd reduced the number of spermatocytes and mature sperms in duck testes (Xia et al. 2016). Excess Mn changed the shape of the testis seminiferous tubules and germ cells and increased white blood cell counts in intercellular space in chicken testes (Du et al. 2015). Pb poisoning induced seminiferous tubules to become shrunken in rat testes (El-Neweshy and El-Sayed 2011). We found that excess Pb led to the deposition of Pb. Our results also indicated that Pb poisoning caused histological changes in the chicken testes.

Overproduction of NO is related to the pathogenesis of many inflammatory diseases, such as poisoning conditions. Atrazine and chlorpyrifos induced NO content and iNOS and resulted in the brain damage of common carps (Wang et al. 2013). Cd induced NO content and iNOS mRNA expression and caused chicken renal injury (Liu et al. 2015b). NO content and iNOS activity increased; and chicken testicular damage occurred after Mn treatment (Liu et al. 2013). Pb exposure induced iNOS activity in brain regions and damaged brains in rats (Govindarajan and Jadhav 2001). In accordance with the previous studies, we found that excess Pb increased NO content and iNOS in the chicken testes. Our results meant that excess Pb caused an inflammatory response in the chicken testes. We also found that there were time-dependent effects on NO content, iNOS activity, and mRNA expression.

NO can stimulate the expression of NF-κB. NF-κB is an important inflammatory factor which can induce the expression of many pro-inflammatory cytokines, including TNF-α, COX-2, iNOS, and PTGEs. TNF-α can also stimulate NF-κB expression, and excess TNF-α can lead to systemic inflammation (Boulanger et al. 2007). The increase of COX-2 may be due to NO stimulating NF-κB in mouse skin (Chun et al. 2004). Our results also demonstrated the previous mechanisms. We found that excess Pb upregulated NF-κB, TNF-α, COX-2, and PTGE mRNA expression and NF-κB protein expression and caused inflammatory response in the chicken testes. Liu et al. (2015c) found that TNF-α could interact with NF-κB in Cd-induced inflammatory response in chicken splenic lymphocytes. The expression of several inflammation factors, including NF-κB, TNF-α, COX-2, and PTGEs, increased, and an inflammatory response occurred in As-exposed chicken gastrointestinal tract (Xing et al. 2015) and in Pb-exposed chicken peripheral blood lymphocytes (Sun et al. 2016). Moreover, in our study, Pb had a time-dependent effect on mRNA expression of all of the previously detected inflammatory factors in the chicken testes.

HSPs are highly conserved cytoprotective proteins and can protect organisms exposed to environmental stressors (Zhao et al. 2013) including heavy metals (Kim et al. 2014). A study found that HSP60 and HSP70 could be the relevant biomarkers of heavy metal poisoning (Mahmood and Jadoon 2014). Cd exposure elevated HSP60 and HSP70 protein levels in gill cells (Ivanina et al. 2008). HSP70 increased after continuous Pb treatment compared with the control group in oribatid mites (Köhler et al. 2005). HSP90 expression increased after Pb poisoning for 1 hour in adult rat oval cells (Agarwal et al. 2009). Zheng et al. (2016) demonstrated that protein levels of HSP70 and HSP90 increased in Pb-exposed chicken cartilage tissue. Our results also indicated that high protein expression of HSPs occurred after Pb exposure. Our results suggested that HSP60, HSP70, and HSP90 were involved in the resistance to Pb-induced toxicity in the chicken testes.

Se could alleviate heavy metal toxicity in testes. Se improved histological changes in silver-exposed rat testes (Ansar et al. 2016) and in Cd-exposed chicken testes (Li et al. 2010). Se could ameliorate Cd-induced oxidative stress, enhance the ability of chicken immune defense system (Zhao et al. 2014b), and relieve Cd-induced kidney toxicity by downregulating NO content and iNOS activity (Liu et al. 2015b). Liu et al. (2015c) found that Se protected against inflammatory responses induced by Cd in chicken splenic lymphocytes through suppressing NF-κB, TNF-α, COX-2, PTGE2, and iNOS expression. Se downregulated HSP70 mRNA level and relieved the toxic effect induced by As poisoning in rat livers (Xu et al. 2013). Consistent with these reports, our results indicated that Se ameliorated Pb deposition; histological changes; and the increase of NO content; iNOS activity; NF-κB, TNF-α, COX-2, PTGEs, and iNOS mRNA expression; and NF-κB, iNOS, HSP60, HSP70, and HSP90 protein expression induced by Pb in the chicken testes. Our results suggested that Se alleviated Pb-induced inflammatory damage and HSP protein expression in the chicken testes. In addition, in our results, Pb decreased the deposition of Se in the chicken testes. We speculate that Se and Pb may form a compound (Patrick 2006; Ahamed and Siddiqui 2007), the compound was excreted, and Pb concentration was decreased in the chicken testes. The mechanism needs to be further studied.

Conclusions

Pb poisoning upregulated NO content; iNOS activity; NF-κB, TNF-α, COX-2, PTGEs, and iNOS mRNA levels in a time-dependent manner; NF-κB, iNOS, HSP60, HSP70, and HSP90 protein levels; and Pb concentration in the chicken testes. Pb poisoning downregulated Se concentration and induced inflammatory damage in the chicken testes. Se alleviated Pb-caused all of the above changes. Se ameliorated Pb-induced inflammatory damage by decreasing inflammatory factors and heat shock proteins in the chicken testes.

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Compliance with ethical standards Informed consent was obtained from all individual participants included in the study.

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

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