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Antagonistic effect of selenium on lead-induced inflammatory injury through inhibiting the nuclear factor- κ B signaling pathway and stimulating selenoproteins in chicken hearts

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Lead (Pb) is a toxic metal and can damage chicken organs. Selenium (Se) is an essential micronutrient and has antagonistic effect on heavy metal toxicity. However, the toxic effect of Pb on inflammatory factors and selenoproteins, and antagonistic effect of Se on Pb toxicity in chicken hearts remain unclear. In the present study, a chicken model to study Pb and Se was established. Seven-day-old male chickens were randomly divided into the control, the +Se group, the +Pb group, and the Se + Pb group. The feeding program of Pb and Se was as follows: lead acetate was added to drinking water and sodium selenite was added to the standard diet. Relative mRNA levels of four inflammatory factors (nuclear factor- κ B (NF- κ B), tumor necrosis factor- α , cyclooxygenase-2, and prostaglandin E synthases) and 25 selenoproteins (including glutathione peroxidase 1 (Gpx1), Gpx2, Gpx3, and Gpx4, thioredoxin reductase 1 (Txnrd1), Txnrd2, Txnrd3, iodothyronine deiodinase 1 (Dio1), Dio2, Dio3, selenoprotein n1 (Sepn1), selenoprotein K (Selk), Sels, Sepw1, Selt, Selh, Selm, 15 kDa selenoprotein, Seli, Selu, Selpb, Sepp1, Selo, Sepx1, selenophosphate synthetase 2 (SPS2)) were detected on the 30th, 60th, and 90th days in chicken hearts. The results indicated that Pb poisoning increased mRNA levels of inflammatory factors, decreased mRNA levels of selenoproteins, and caused histological changes in the chicken hearts. Se alleviated Pb-caused all the above changes in the chicken hearts. Our results suggested that Se supplementation alleviated Pb-induced inflammatory injury through inhibiting the NF- κ B signaling pathway and stimulating selenoproteins in the chicken hearts.

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1. Introduction

Lead (Pb) is a toxic metal and exists in natural environment. Pb-contaminated fodder and water pose potential risks to humans through the food chain. The concentration of Pb increased in cattle and sheep living near a Pb-polluted mining area.¹ Pb has a negative influence on the central nervous system,² and the behavior and mental development of children.³ Pb has adverse effects on animals, such as rat kidneys,⁴ chicken livers,⁵ and cartilage tissue⁶ and Pb induced cardiovascular toxicity in rats.⁷ A previous study indicated that Pb exposure activated

cyclooxygenase-2 (COX-2) in rat vascular smooth muscle cells.⁸ Inflammation is a characteristic of the clinical syndrome of heart failure.⁹ Research has investigated the roles of inflammatory markers such as tumor necrosis factor- α (TNF- α) and nuclear factor- κ B (NF- κ B) in cardiovascular diseases.¹⁰ TNF- α could serve as a potential bio-marker for coronary heart disease.¹¹ NF- κ B regulates the expression of TNF- α .¹² Prostaglandin E2 (PGE2) is a principal mediator of inflammation in diseases.¹³ Arachidonic acid is transformed into PGE2 *via* cyclooxygenase enzymes in cells.¹⁴ Therefore, NF- κ B signaling pathways are important in heart diseases. A study also found that exposure to cadmium (Cd) increased TNF- α in Wistar rat heart tissues.¹⁵ However, the mechanism of inflammatory response caused by Pb is still unclear in chicken hearts. Therefore, we investigated the effect of Pb on NF- κ B signaling pathway in chicken hearts.

Selenium (Se) is an essential micronutrient and can antagonize different heavy metal toxicities¹⁶ and hepatocellular carcinoma.¹⁷ Se plays an important role in maintaining normal cardiovascular function. Many of its physiologic roles are directly attributed to its presence within selenoproteins in the form of the amino acid selenocysteine.¹⁸ Studies have shown

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that Se deficiency can cause heart damage, such as Keshan disease¹⁹ and Chagas' disease.²⁰ However, antagonistic effect of Se on Pb toxicity is still unclear in chicken hearts.

Effect of Pb on mammals and chickens has recently attracted considerable attention. For example, Hao Wang *et al.*²¹ studied that Se alleviated Pb-induced increase of relative mRNA expression of inflammation factors and heat shock proteins in chicken livers. However, inflammatory injury induced by Pb and protective effect of Se on Pb poisoning in chicken hearts remain unclear. The aim of the present study was to explore antagonistic effect of dietary Se on inflammatory injury induced by Pb toxicity through measuring mRNA levels of inflammatory factors and selenoproteins, and to provide theoretical basis of remission effect of Se on heart injury induced by Pb in chickens.

2. Materials and methods

2.1 Animal model and tissue sample

One hundred and eighty Hyline male chickens (1 day-old) were acclimated for 7 days and then randomly divided into four groups. The control group was fed basic diet (0.49 mg kg⁻¹ Se) and drinking water. The +Se group was fed sodium selenite (Na₂SeO₃, analytical reagent grade, Tianjinzhiyuan, Chemical Reagent Co. Ltd., Tianjin, China)-added the basic diet (1 mg kg⁻¹ Se) and drinking water. The +Pb group was fed lead acetate

((CH₃OO)₂Pb, analytical reagent grade, Tianjinzhiyuan Chemical Reagent Co. Ltd., Tianjin, China)-added drinking water (350 mg L⁻¹ Pb), according to median lethal dose (LD₅₀) for Pb in chickens²² and the need of the chicken experiment in toxicology.²³ The Se + Pb group was fed Na₂SeO₃-added the basic diet (1 mg kg⁻¹ Se) and (CH₃OO)₂Pb-added drinking water (350 mg L⁻¹ Pb). The chickens were maintained in the Laboratory Animal Center, College of Veterinary Medicine, Northeast Agricultural University, China. Food and water were provided *ad libitum*.

On the 30th, 60th, and 90th days of the experiment, fifteen chickens in each group were randomly selected, and euthanasia was performed. Heart tissues were quickly removed, rinsed with ice-cold, sterile deionized water. Each heart was divided into two parts. One part was fixed with 5% paraformaldehyde for histological observation. Another part was frozen in liquid nitrogen and stored at -80 °C until required. 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.

2.2 Relative mRNA levels of inflammatory factors and selenoproteins

2.2.1 Primer synthesis. The primer sequences of inflammatory factors and selenoproteins published in GenBank were

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

Gene	Serial number	Forward primer (5' → 3')	Reverse primer (5' → 3')
NF-κB	NM_205134	TCAACGCAGGACCTAAAGACAT	GCAGATAGCCAAGTTCAGGATG
TNF-α	NM_204267	GCCCTTCCTGTAACCAGATG	ACACGACAGCCAAGTCAACG
PTGEs	NM_001194983	GTTCTGTTCATTCGCTTCTAC	CGCATCCTCTGGGTTAGAC
COX-2	NM_001167718	TGTCCTTTCAGTCTTCCAT	TCCATTGCTGTGTTTGAGGT
GPx1	BX935086.2	ACGGCGCATCTTCCAAG	TGTTCCCAACCACTTCTCT
GPx2	NM_001277854.1	ACGGCACCAACGAGGAGAT	TTCAGGTAGGCGAAGACGG
GPx3	NM_001163232.1	CCTGCAGTACCTCGAAGTGA	CTTCAGTGCAGGGAG GATCT
GPx4	AF498316	CTTCGTCTGCATCATCACCAA	TCGACGAGCTGAGTGTAAATCAC
Dio1	Y11110.1	GCGCTATACCACAGGCGAGTA	GGTCTTGCAAATGTCACCAC
Dio2	AF125575.1	ATTTGCTGATCAGCTTCCAG	GCTCAGAAACAGCACCATGT
Dio3	Y11273.1	CTGTGCATTCGCAAGAAGAT	GCCGACTTGAAGAAGTCCAG
Txnrd1	NM_001030762.2	TACGCCTCTGGGAAATTCGT	CTTGCAAGGCTTGTCCCAGTA
Txnrd2	NM_001122691.1	GCTCTTAAAGATGCCAGCACTAC	GAACAGCTTGAGCCATCACAGA
Txnrd3	NM_001122777.1	CCTGGCAAACGCTAGTTGTG	CGCACCATTACTGTGACATCTAGAC
Sepr1	NM_001114972.1	CAGGATCCATGCTGAGTTCCA	GAGAGGACGATGTAACCCGTAAC
Selk	NM_001025441.2	GAAGAGGGCTCCAGGAAAT	CAGCCATTGGTGGTGGACTAG
Sels	NM_001024734.2	GCGTCGCCATCTATCTCATCGT	TCTTCTGCCCTCGCTTCTGTTCTT
Selw1	NM_001166327.1	TGGTGTGGGTCTGCTTTACG	CCAAAGCTGGAAGGTGCAA
Selt	NM_001006557.3	AGGAGTACAT GCGGG TCATCA	GACAGACAGGAAGGATGCTATGTG
Selh	BX932816.2	CATCGAGCACTGCCGTAG	GACACCTCGAAGCTGTTCTCT
Selm	CR390234.1	AAGAAGGACCACCCAGACCT	GCTGTCTGTCTCCCTC ATC
Sep15	NM_001012926.2	ACTTGGCTTCTCCAGTAACCTGCT	GCCTACAGAATGGATCCAACCTGA
Seli	XM_426086.1	TGCCAGCCTCTGAACCTGGAT	TGCCAAACCCAGACATCACCAT
Selu	NM_001193518.1	GATGCTTTTCAGGCTTCTTCC	CTGTCTTCTGCTCCAATCA
Selpb	XM_003641687.1	AGGCCAACAGTACCATGGAG	GTGGTGAGGATGGAGATGGT
Sepp1	NM_001031609	CCAAGTGGTCAGCATTCACATC	ATGACGACCACCTCAGCAT
Selo	NM_001115017.1	CCAGCGTTAACCGGAATGAT	ATGCGCCTCTGGATTTCT
Sepr1	NM_001135558.1	TGGCAAGTGTGGCAATGG	GAATTTGAGCGAGCTGTGAAT
SPS2	NM_001187543.1	CGTTGGGTATCGGAACCTGAC	CGTCCACCAGAGGGTAGAAA
GADPH	K01458	AGAATCATATCCAGCGT	AGCCTTCACTACCCTCTTG



showed in Table 1. GAPDH was used as an internal reference gene. The primers were synthesized by Invitrogen Biotechnology Co. Ltd., Shanghai, China.

2.2.2 Total RNA isolation and reverse transcription. The extraction of total RNA was performed using TRIzol reagent according to the manufacturer's instructions (Invitrogen, China). The dried RNA pellets were resuspended in 40 μ L of diethylpyrocarbonate-treated water. The concentration and purity of the total RNA were determined spectrophotometrically (Healthcare Bio-Sciences AB, Sweden) at 260/280 nm. Reverse transcription (RT) reactions (60 μ L) contained 6 μ L of total RNA, 1.5 μ L of RNase inhibitor, 3 μ L of dNTP mixture (10 mM each), 3 μ L of 20 \times oligo dT (25), 3 μ L of golden M-MLV RT, 6 μ L of 10 \times RT buffer, and 37.5 μ L of RNase free H₂O. The RT procedure carried on three times of water bath heating, at 30 $^{\circ}$ C for 15 min, at 55 $^{\circ}$ C for 50 min, and at 85 $^{\circ}$ C for 10 min. The cDNA was diluted five times with sterile water and stored at -20 $^{\circ}$ C until used.

2.2.3 Quantitative real-time PCR. Quantitative real-time PCR was performed using Light Cycler[®] 96 System according to the manufacturer's instruction (Roche, Switzerland). Reactions were performed in a 10 μ L reaction mixture containing 5 μ L of 2 \times SYBR green PCR master mix (Roche, Switzerland), 1 μ L of diluted cDNA, 0.3 μ L of each primer (10 μ M), and 3.4 μ L of PCR-grade water. The PCR procedure consisted of heating the reaction mixture to 52 $^{\circ}$ C for 2 min and 95 $^{\circ}$ C for 10 min followed by 40 cycles of 95 $^{\circ}$ C for 15 s and 60 $^{\circ}$ C for 1 min, 95 $^{\circ}$ C for 15 s, and 60 $^{\circ}$ C for 20 s. The melting curve analysis showed only one peak for each PCR product. Electrophoresis was performed with the PCR products to verify primer specificity and product purity. A dissociation curve was run for each plate to confirm the production of a single product. The experiment was repeated 3 times for each sample. Relative mRNA abundance was calculated according to the method of Pfaffl.²⁴

2.3 Histological observation

The tissues were placed in 5% paraformaldehyde solution. Paraffin-embedded issue block was performed. Each issue block was sliced into 5 μ m-thick sections. The sections were mounted on glass and stained with hematoxylin and eosin. The slides were observed under a light microscope (Eclipse 80i, Nikon, Tokyo, Japan).

2.4 Statistical analysis

Statistical analyses of all data were carried out with two-way analysis of variance (ANOVA) using SPSS (version 13; SPSS Inc., Chicago, IL, USA) for Windows. The comparisons for groups were verified by nonparametric as Kruskal-Wallis ANOVA test and Mann-Whitney *U* test. The data were presented as means \pm standard deviation (bars, *n* = 5). Statistically significant differences: bars with different uppercase letters within the same group at different time points are significantly different (*P* < 0.05), and bars with different lowercase letters in the different groups at the same time point are significantly different (*P* < 0.05).

3. Results

3.1 NF- κ B, TNF- α , COX-2, and PTGES mRNA expression

Relative mRNA expression of inflammatory factors (NF- κ B (Fig. 1a), TNF- α (Fig. 1b), COX-2 (Fig. 1c), and PTGES (Fig. 1d)) was shown in Fig. 1. All the inflammatory factors in the +Pb group were significantly higher (*P* < 0.05) than that in the control, +Se, and Se + Pb groups. And all the inflammatory factors in the Se + Pb group were significantly higher (*P* < 0.05) than those in the control and +Se groups. NF- κ B, TNF- α , and COX-2 increased significantly (*P* < 0.05) with the increase of treatment time in the +Pb group. PTGES on the 90th day was significantly higher (*P* < 0.05) than that on the 30th and 60th days

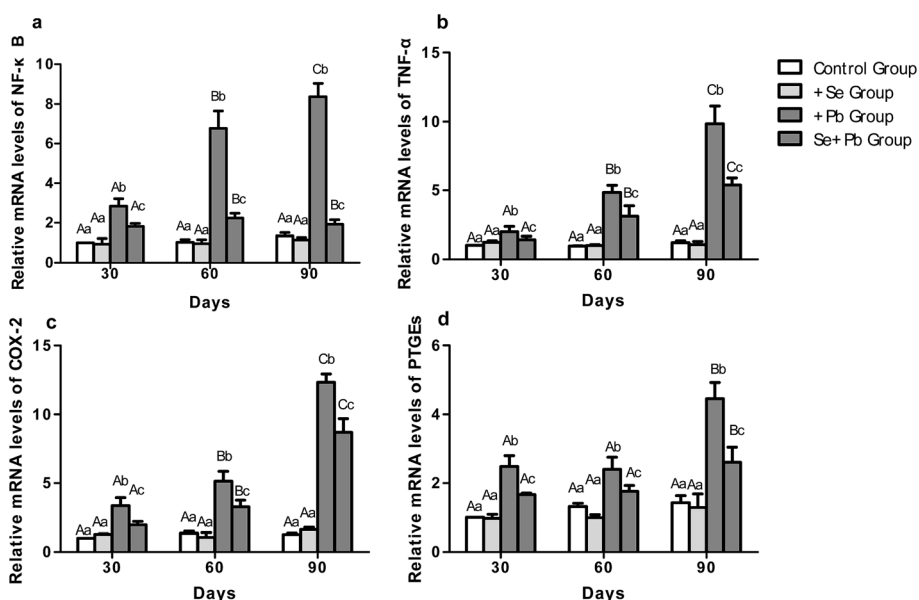


Fig. 1 NF- κ B, TNF- α , COX-2 and PTGES mRNA levels in chicken hearts. Fifteen chickens consisted of five replicate pens, with each pen containing three chickens.



in the +Pb and Se + Pb groups. NF- κ B on the 60th and 90th days was significantly higher ($P < 0.05$) than that on the 30th day in the Se + Pb group. TNF- α and COX-2 increased significantly ($P < 0.05$) with the increase of treatment time.

3.2 Gpx1, Gpx2, Gpx3, and Gpx4 mRNA expression

Glutathione peroxidase 1 (Gpx1), Gpx2, Gpx3, and Gpx4 mRNA expression was shown in Fig. 2. Relative mRNA levels of all the Gpxs in the +Pb group were significantly decreased ($P < 0.05$) compared with those in the control, +Se, Se + Pb groups except Gpx1 and Gpx4 on the 30th and 60th days, and Gpx3 on the 30th day. All the Gpxs in the Se + Pb group were significantly decreased ($P < 0.05$) compared with those in the control and +Se

groups except Gpx3 on the 90th day. In the +Pb group, Gpx1 on the 60th and 90th days was significantly decreased ($P < 0.05$) compared with that on the 30th day. Gpx2 and Gpx3 decreased significantly ($P < 0.05$) with the increase of treatment time. Gpx4 on the 90th day was significantly increased ($P < 0.05$) compared with that on the 30th and 60th days.

3.3 Thioredoxin reductases and iodothyronine deiodinases mRNA expression

Relative mRNA expression of thioredoxin reductases (Txnrds) and iodothyronine deiodinases (Dios) was shown in Fig. 3. Txnr1, Txnr2, Txnr3, Dio1, Dio2, and Dio3 in the +Pb group were significantly lower ($P < 0.05$) than those in the control, +Se,

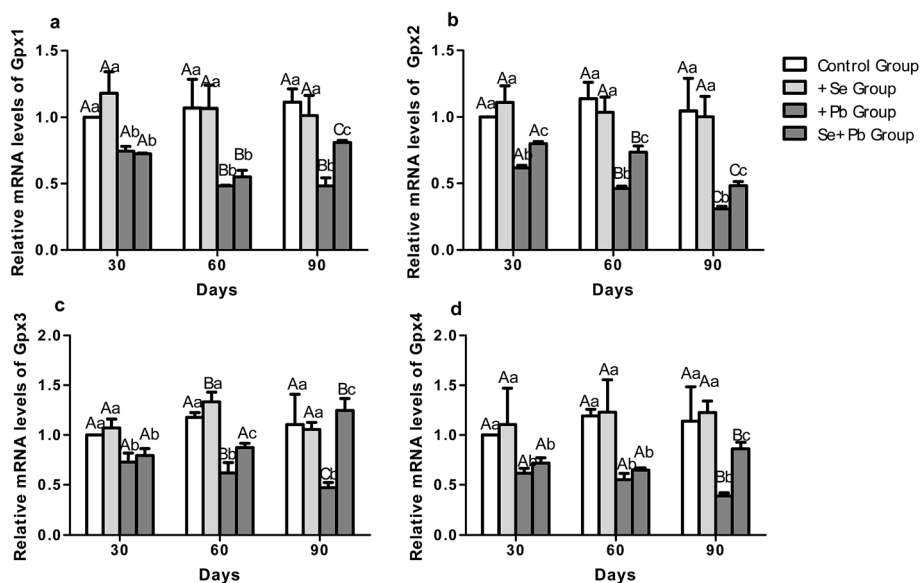


Fig. 2 Gpx1, Gpx2, Gpx3, and Gpx4 mRNA levels in chicken hearts. Fifteen chickens consisted of five replicate pens, with each pen containing three chickens.

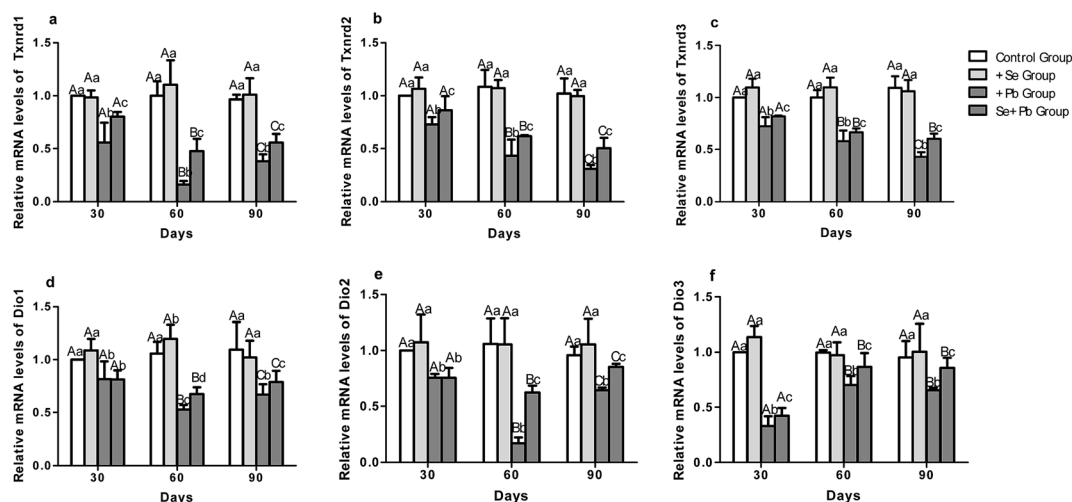


Fig. 3 Txnrds and Dios mRNA levels in chicken hearts. Txnr1, Txnr2, Txnr3, Dio1, Dio2 and Dio3 were showed. Fifteen chickens consisted of five replicate pens, with each pen containing three chickens.



and Se + Pb groups except Dio1 and Dio2 on the 30th day. Txnrds and Dios in the Se + Pb group were significantly lower ($P < 0.05$) than those in the control and +Se groups.

3.4 Relative mRNA expression of 15 selenoproteins

Relative mRNA expression of 15 selenoproteins was detected. As shown in Fig. 4, selenoprotein n1 (Sepn1), selenoprotein K (Selk), Sels, Sepw1, Selt, Selh, Selm, 15 kDa selenoprotein (Sep15), Seli, Selu, Selpb, Sepp1, Selo, Sepx1, selenophosphate synthetase 2 (SPS2) in the +Pb group were significantly decreased ($P < 0.05$) compared with those in the control, +Se, and Se + Pb groups. All the selenoproteins in the Se + Pb group were significantly decreased ($P < 0.05$) compared with those in the control and +Se groups.

3.5 Histological observation

Histological results were shown in Fig. 5. In the control group on the 90 day, histology of chicken heart tissue displayed

normal structure with regular morphology (Fig. 5a). In the +Pb group on the 30th day (Fig. 5b), there was lightly edema in cardiac muscle cells. In the +Pb group on the 60th day (Fig. 5c), cardiac muscle cells had edema. Muscle fiber pulp had dissolved. Cell hypertrophy and vacuole degeneration were observed. In the +Pb group on the 90th day (Fig. 5d), cardiac muscle cells occurred focal necrosis and inflammatory cell infiltration. In the Se + Pb group (Fig. 5e) on the 90th day, some cardiac muscle fibers were slightly disorganized. Some cardiac muscle cells occurred inflammatory cell infiltration.

4. Discussion

Pb can cause inflammatory injury in testicular macrophages of male Swiss albino mice.²⁵ Inflammatory processes play critical roles in the treatment of heart failure.²⁶ NF- κ B activation could cause cardiomyopathy and heart failure by evoking an excessive inflammatory response in cardiomyocytes.²⁷ NF- κ B regulates

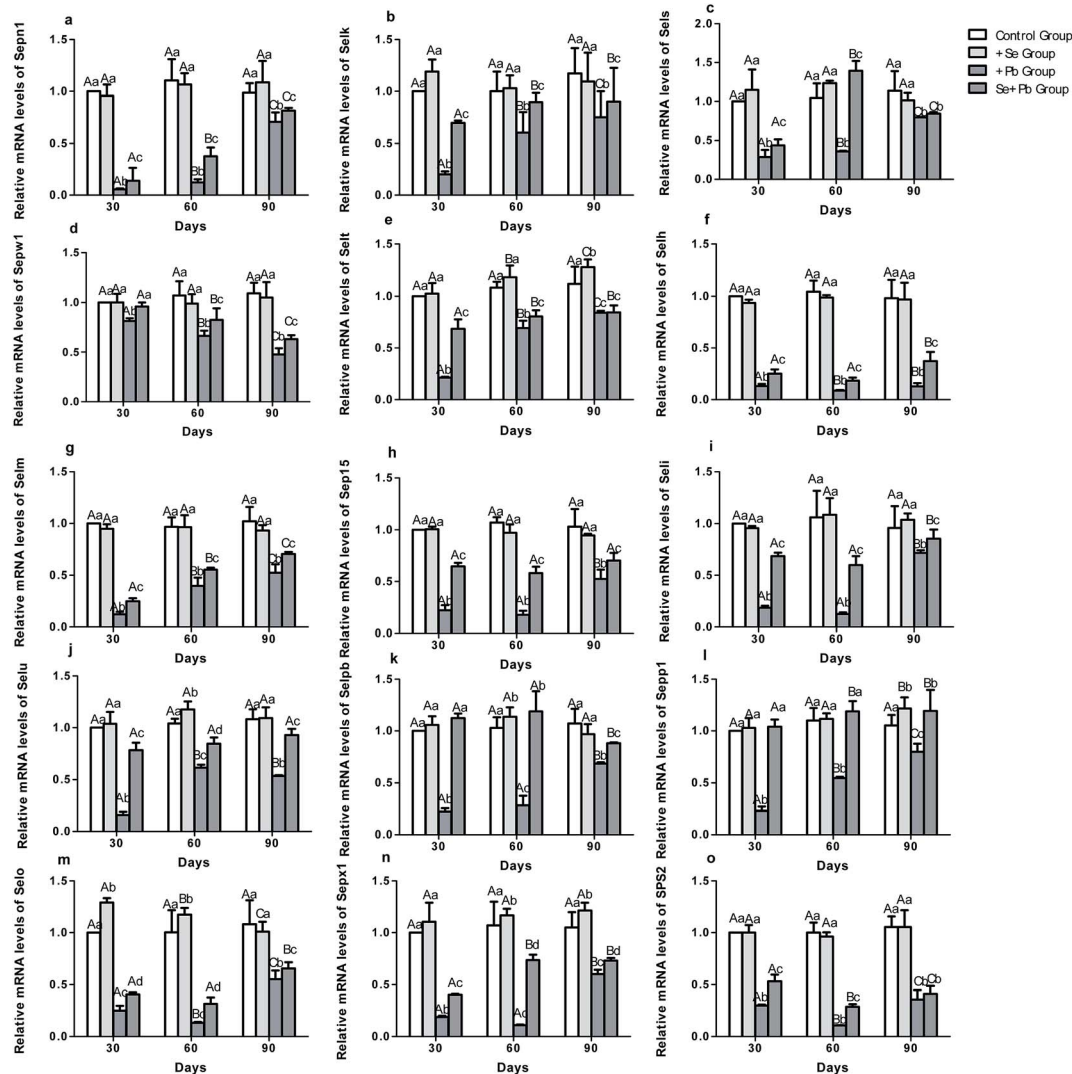


Fig. 4 Relative mRNA expression of 15 selenoproteins in chicken hearts. Fifteen chickens consisted of five replicate pens, with each pen containing three chickens.



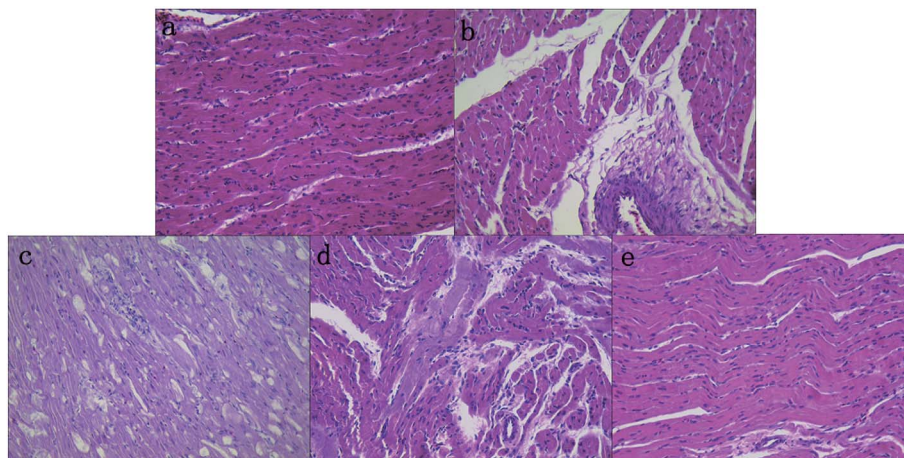


Fig. 5 Histological observations in chicken heart of dietary Se against Pb-induced inflammatory injury ($\times 400$). (a): The control group on the 90th day. (b–d): The +Pb group on the 30th, 60th, and 90th days. (e): The Se + Pb group on the 90th day.

proinflammatory cytokines in both innate and adaptive immunity.²⁸ NF- κ B was involved in the regulation of COX-2 and TNF- α in Pb-induced immunotoxicity and inflammatory response.²⁹ Excess Pb can induce abnormal microgliosis and astrogliosis in young mice through triggering NF- κ B signaling cascades.³⁰ Mingwei Xing *et al.*³¹ reported that arsenic induced inflammation response and may cause chicken digestive function regression of gastrointestinal tract by affecting inflammation-related genes. Excess Pb increased COX-2 and PGE2 in human aorta vascular smooth muscle cells.³² Pb can cause inflammatory damage in the peripheral blood lymphocytes of chickens.³³ These findings were consistent with our study. Our results indicated that excess Pb increased NF- κ B, PTGES, TNF- α , and COX-2 mRNA expression (see Fig. 1) and inflammatory injury (see Fig. 5b–d) in the chicken hearts. Our results indicated that excess Pb induced inflammatory injury through inhibiting NF- κ B signaling pathway in the chicken hearts.

Previous studies showed that Se has antagonistic effect on toxic heavy metals. Se exerts a protective role against Cd-induced inflammatory cytokines in chicken splenic lymphocytes³⁴ and Pb-induced inflammatory cytokines in chicken peripheral blood lymphocytes.³³ Our study was similar to these studies. In our study, we found that NF- κ B, TNF- α , COX-2, and PTGES mRNA levels decreased in the Se + Pb group compared to those in the +Pb group (see Fig. 1). Degree of inflammatory injury in the Se + Pb group was lower than that in the +Pb group (see Fig. 5d and e). Our results indicated that Se antagonized Pb-induced inflammation injury in the chicken hearts.

Selenoproteins are main indicators for some functions of Se in animals. Some selenoproteins are very sensitive to dietary Se in some chicken tissues.^{35,36} Se deficiency caused the decline of Gpxs activity in chicken immune organs.³⁷ Se deficiency influenced selenoprotein gene expression in chicken muscles.³⁸ Gpx1, Gpx2, Gpx3, Gpx4, Txnrd1, Txnrd2, Txnrd3, Dio1, Dio2, Dio3, Sepn1, Selk, Sels, Sepw1, Selt, Selh, Selm, Sep15, Seli, Selu, Selpb, Sepp1, Selo, Sepx1, SPS2 exist in chickens. These selenoproteins have anti-inflammatory properties and play important roles in inflammatory response,³⁹ immune response,⁴⁰ and

apoptosis.⁴¹ One study showed that excess Pb decreased GPx in rat livers and erythrocytes.⁴² Our results indicated that excess Pb decreased mRNA levels of the 25 selenoproteins (see Fig. 2–4). Dietary Se supplementation alleviated the decrease of Gpx3, Txnrd1, Txnrd2, Txnrd3, Dio2, and Seln mRNA levels induced by aflatoxin B1 in male avian broiler livers.⁴³ Zhao *et al.*⁴⁴ found that Se played protective role by increasing mRNA expression of Selk, Seln, Sels, and Selt against Cd in chicken splenic lymphocytes. Gao *et al.*⁴⁵ reported that Se alleviated the decline of selenoprotein gene expression induced by Pb in chicken cartilages. In accordance with the above researches, our results indicated that Se alleviated the decline of Gpx2, Gpx3, Gpx4, Txnrd1, Txnrd3, Txnrd3, Dio1, Dio3, Dio3, Sepn1, Selk, Sels, Sepw1, Selt, Selh, Selm, Sep15, Seli, Selu, Selpb, Sepp1, Selo, Sepx1, SPS2 mRNA levels induced by Pb in the chicken hearts.

5. Conclusions

In conclusions, excess Pb increased mRNA levels of NF- κ B, TNF- α , COX-2, and PTGES; and decreased mRNA levels of Gpxs, Txnrds, Dios, Sepn1, Selk, Sels, Sepw1, Selt, Selh, Selm, Sep15, Seli, Selu, Selpb, Sepp1, Selo, Sepx1, and SPS2 in the chicken hearts. Excess Pb changed histology in the chicken hearts. Se alleviated the changes of histology and all the above inflammatory factors and selenoproteins caused by Pb in the chicken hearts. Se supplementation alleviated Pb-induced inflammatory injury through inhibiting NF- κ B signaling pathway and stimulating selenoproteins in the chicken hearts.

Conflict of interest

The authors declare that there are no conflicts of interest.

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