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Ameliorative Effect of Selenomethionine on Cadmium-Induced Hepatocyte Apoptosis via Regulating PI3K/AKT Pathway in Chickens

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Abstract

Selenium (Se) is a trace element for human and animal health. Cadmium (Cd) is a known human carcinogen. The effects of Cd on the environment and humans are well known. Because chickens are at the top of the food chain, it is a good experimental animal model for assessing heavy metal toxicity and its potential threat to humans. Selenomethionine (Se-met) is a suitable form for nutritional Se supplementation. Therefore, the toxicity of Cd to the chicken liver and the antagonistic effects of Se-met on Cd were examined at the molecular level in the present study. The results showed that oxidative stress indicators (apoptosis-related genes, P13K/AKT pathway–related genes, and heat shock proteins (HSPs)–related genes) in the Cd group have changed significantly, indicating Cd induced hepatocyte stress and apoptosis. Interestingly, the changes in oxidative stress indicators (apoptosis-related genes, P13K/AKT pathway–related genes, and HSPs-related genes) in the Cd-Se-met group were mitigated compared with the control group. Our results indicated that Cd can induce hepatocyte apoptosis and stress in the chickens. Se-met has an ameliorative effect on Cd-induced apoptosis of chicken hepatocyte by regulating PI3K/AKT pathway. Our findings will provide a new insight for better understanding of the detoxification function of Se-met to heavy metals.

Keywords Selenomethionine · Cadmium · Oxidative stress · Apoptosis · P13K/AKT pathway · HSPs

Introduction

Cadmium (Cd) is a typical environmental pollutant that can cause pollution of water, air, and soil [1, 2]. Cd, as a non-essential element of the human body, can enter the human body through occupational exposure, breathing, and eating food contaminated with Cd, and reach various organs of the body through blood circulation, thus endangering human health [3]. The "Itai-itai disease" in Toyama, Japan, and the "Cd Rice incident" in Hunan, China, were caused by Cd contamination in the water environment. Cd has been listed as a key food pollutant by the World Health Organization and has been identified as class I carcinogen by the International Agency for Research on Cancer [4]. Severe cases are accompanied by pulmonary edema and damage of the liver and kidney. Chronic Cd poisoning mainly causes kidney, lung, liver, tooth, and bone damage [5]. Some studies have found that Cd is toxic to many animals, including aquatic animals, mammals, reptiles, and birds [1, 6, 7]. Since Cd can accumulate in animal tissues, its toxicity can be amplified through the food chain, posing a threat to humans [6]. For this reason, the study of Cd toxicity to organisms at the top of the food chain has been widely concerned by researchers [1, 5, 6].

Selenium (Se) is an essential trace element in animals and humans [8]. Because Se is an important part of some antioxidase and selenoproteins, it plays an important biological function in the body [9–11]. Clinical medicine proves that Se deficiency will directly lead to the decline of human immunity. More than 40 diseases that threaten human health and life are related to Se deficiency in the human body, such as cancer, cardiovascular diseases, liver diseases, cataracts, pancreatic diseases, diabetes, and reproductive system diseases [12]. Studies have confirmed that the biological functions of Se mainly

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include balancing redox reactions, improving immunity, detoxification, and preventing cancer [13, 14]. Selenomethionine (Se-met) is an organic form of Se and is suitable for nutritional Se supplementation [15]. Studies found that the decrease in thyroid antibody titration was particularly pronounced in subjects received 200 mg of Se-met daily, suggesting that the supplementation of Se-met enhanced the role of thyroid autoimmunity [16, 17]. The effects of heavy metals on the environment and humans are well known. Environmental researchers have long been hoping to find a real substance that can reduce the toxicity of heavy metals. Because Se plays an extensive biological role in organisms, the antagonistic effect of Se on the toxicity of heavy metals has been a hot spot in the field of environmental toxicology in recent years. A study showed that Se supplementation can alleviate the decrease in selenoprotein mRNA level and the Th1/Th2 imbalance in peripheral blood lymphocytes caused by lead, indicating that Se has an antagonistic effect on lead toxicity [18]. Rahman et al. found that Se can regulate arsenic-induced intrinsic apoptosis pathway through enhancement of mTOR/AKT autophagy signaling pathway by employing antioxidant potentials and inhibiting cellular accumulation of arsenic in PC12 cells, indicating that Se has an ameliorative effect on arsenic-induced cytotoxicity in PC12 cells via modulating autophagy/apoptosis [19].

Cd has non-degradable properties and long half-life. It can accumulate and produce toxic effects in many organs and tissues such as the kidneys, testicles, liver, lungs, brain, and bones of animals [20–22]. The liver is an important metabolic organ in animals and participates in the synthesis and metabolism of various substances such as glycogen, cholesterol, lipoproteins, and plasma proteins. Unlike mammals, poultry liver is also the most important site for lipid synthesis and is closely related to lipid metabolism. More importantly, the poultry liver is not only an important detoxification organ in the body but also one of the target organs for Cd accumulation [23, 24]. Because chickens are at the top of the food chain, it is a good experimental animal model for assessing heavy metal toxicity and its potential threat to humans. Se-met is a suitable form for nutritional Se supplementation. Although some studies have been conducted on the biological effects of Se-met, there is currently less research on the antagonistic effects of Se-met on heavy metal toxicity, especially environmental pollutant Cd. Therefore, the toxicity of Cd to the chicken liver and the antagonistic effects of Se-met on Cd were examined at the molecular level in the present study.

Materials and Methods

Animals and Treatment

All experiments were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University. One hundred and twenty healthy Hailan white broilers (1-day-old) were randomly divided into four groups: control group, Se-met group, Cd group, and Cd-Se-met group. The chickens in the control group were feed basal diets with sodium selenite (per kilogram of base diet contains 0.3 mg Se). The chickens in the Se-met group were feed basal diets with Se-met (the Se content is 0.3 mg/kg). The chickens in the Cd-Se-met group were feed basal diets with Cd and Se-met (Cd, 150 mg/kg; Se, 0.3 mg/kg). The chickens in the Cd group were feed basal diets with Cd (Cd, 150 mg/kg). The composition of the basic diet for broilers is shown in Table 1. All broilers were given ad libitum access to food and water. On the thirty-fifth day of the experiment, the chickens were euthanized. The liver tissues were divided into two parts. One part was fixed in 10% formalin and embedded in paraffin for TUNEL assay, and the other part was stored at − 80 °C for further use.

Determination of TUNEL

In order to investigate whether Cd and Se-met could affect liver apoptosis, TUNEL analysis was performed using an in situ cell death detection kit (fluorescein, Roche, Basel, Switzerland). TUNEL staining shows DNA fragmentation and is recognized as a standard technique for the detection of apoptosis in tissue sections. First, the tissue sections were prepared according to normal methods. The obtained sections were then operated according to the instructions of the kit. The resulting sections were observed under a fluorescence microscope for anti-fluorescence quenching. Three sections were selected randomly from each sample and the numbers of positive cells were counted for each section.

Determination of Oxidative Stress Index

Liver homogenization was prepared under ice field conditions. The activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and nitric oxide synthase (iNOS), and the contents of malondialdehyde (MDA) and nitric oxide (NO) were measured according to the detection kit instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Determination of Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from each sample using Trizol reagent (Invitrogen, Carlsbad, CA). The complementary DNA (cDNA) was synthesized according to the method reported by Xing et al. [25]. The expression level of each gene was quantified by a Light Cycler® 480 System (Roche, Basel, Switzerland) using Fast Universal SYBR Green Master (Roche). The primer for each gene was designed using

Table 1 The composition of the basic diet for broilers

Composition	First stage (%)	Second stage (%)	Third stage (%)
Corn	56.3	61	64.8
Soybean meal	37	28	23
Rice brain	0	1.42	2.8
Vegetable oil	1.7	2	2.2
Zeolite powder	1.4125	3.9925	3.6125
NaC1	0.25	0.25	0.25
$CaHPO_4$	1.4	1.4	1.4
Choline	0.12	0.12	0.12
Stone powder	1.3	1.3	1.3
Multiple trace elements	0.02	0.02	0.02
Methionine	0.16	0.16	0.16
Lysine (%)	0.06	0.06	0.06
Phytase	0.015	0.015	0.015
Bacitracin zinc	0.05	0.05	0.05
Compound enzyme	0.01	0.01	0.01
Trace elements	0.2	0.2	0.2
Antioxidants	0.0025	0.0025	0.0025

Primer Premier Software 5.0 based on the deposited sequences in GenBank (Table 2). The β-actin was used as an internal reference. The change in gene expression was determined using the $2^{-\Delta\Delta Ct}$ method.

Western Blot Analysis

Western blot analysis was executed according to the method reported by Hu et al. [26] with some modifications. Briefly, total protein from each sample was separated by 12% SDS-PAGE electrophoresis. Protein was transferred to the PVDF membrane under ice conditions for 90 min. The membrane was blocked with 5% skim milk for 2 h at 37 °C and then incubated with primary antibody for 12 h at 4 °C. Subsequent to washing three times with PBST for 10 min, the membrane was incubated with peroxidase-conjugated secondary antibody against rabbit IgG for 1 h at room temperature. Protein levels were determined by Image VCD Gel Imaging Software

Table 2 Gene-specific primers for qRT-PCR

Gene	Forward	Reverse
NF-κB	5-TCAACGCAGGACCTAAAGACAT-3	5-GCAGATAGCCAAGTTCAGGATG-3
AKT	5-ATGGAATGGACGAAGAGG-3	5-ATCCGTGGACGATACTGG-3
PI3K	5-CTTTTCTGACCCGCTGACTTT-3	5-AATTTCTTACCCACCGCTTC-3
Caspase-3	5-CTGAAGGCTCCTGGTTTA-3	5-TGCCACT CTGCGATTTAC-3
Caspase-9	5-ATTCCTTTCCAGGCTCCATC-3	5-CACTCACCTTGTCCCTCCAG-3
P53	5-GAGATGCTGAAGGAGATCAA TGAG-3	5-GTGGTCAGTCCGAGCCTTTT-3
Bak	5-GCGCAGGCCATCACGAGAGA-3	5-CCGGCCCCAGTTAATGCCGC-3
Bax	5-TCCATTCAGGTTCTCTTGACC-3	5-GCCAAACATCCAAACACAGA-3
Cyt C	5-CTTCTTCCTCCTGGTGAACG-3	5-GCACTCCGAAAGTCTCCTGA-3
Bcl-2	5-ATCGTCGCCTTCTTCGAGTT-3	5-ATCCCATCCTCCGTTGTTCT-3
HSP27	5-ACACGAGGAGAAACAGGATGAG-3	5-ACTGGATGGCTGGCTTGG-3
HSP40	5-GGGCATTCAACAGCATAGA-3	5-TTCACATCCCCAAGTTTAGG-3
HSP60	5-AGCCAAAGGGCAGAAATG-3	5-TACAGCAACAACCTGAAGACC-3
HSP70	5-CGGGCAAGTTTGACCTAA-3	5-TTGGCTCCCACCCTATCTCT-3
HSP90	5-TCCTGTCCTGGCTTTAGTTT-3	5-GTGCCCACGCTGTGCTTAC-3
β-Actin	5-CCGCTCTATGAAGGCTACGC-3	5-CTCTCGGCTGTGGTGGTGAA-3

(National Institutes of Health, Bethesda, MD) and β-actin (1:1000) was used as internal control.

Statistical Analysis

The statistical analysis of all data was performed using SPSS for Windows (version 21, SPSS Inc., Chicago, IL). All values are expressed as means ± standard deviation. One-way ANOVA with a paired *t* test was used to elucidate if there were significant differences between the MA/HA groups and the LA group. Tukey's paired test was used to determine significant differences between different times in the same concentration group. $P < 0.05$ was considered significant.

Results

TUNEL Analysis in the Broiler Liver

To understand the effect of Cd on hepatocellular apoptosis in poultry and the protective effect of Se-met on Cd-induced apoptosis, we carried out TUNEL assay in the liver of broilers. The results of the TUNEL assay are shown in Fig. 1. The number of TUNEL positive nuclei in the Cd-Se-met group and the Cd group increased significantly ($P < 0.05$) compared with the control group (Fig. 1A, C, D, E). A significant decrease ($P < 0.05$) in the number of TUNEL-positive nuclei was observed in the Cd-Se-met group compared with the Cd group (Fig. 1C, D).

Oxidative Stress Analysis in the Broiler Liver

To understand the effect of Cd on liver oxidative stress in poultry and the antioxidant effect of Se-met on Cd-induced oxidative stress, the antioxidant index was analyzed in the liver of broilers (Fig. 2). The activity of SOD, CAT, and GSH-Px in the Cd-Se-met group and the Cd group decreased significantly ($P < 0.05$) compared with the control group. On the contrary, MDA levels in the Cd-Se-met group and the Cd group increased significantly ($P < 0.05$) compared with the control group. There was no significant change ($P > 0.05$) in the antioxidant index of the Se-met group compared with the control group.

Analysis of NO Content and iNOS Activity

NO content and iNOS activity in the liver of broilers are shown in Fig. 3. Compared with the control group, the activity of iNOS in the Cd-Se-met group and the Cd group increased ($P < 0.05$) by 33.52 % and by 71.13 %, respectively. Compared with the Cd group, the activity of iNOS was significantly inhibited ($P < 0.05$) in the Cd-Se-met group, indicating that Se-met has a certain protective effect on Cd-induced liver damage. A significant increase ($P < 0.05$) in the NO content was observed in the Cd-Se-met group and the Cd group compared with the Cd group. Compared with the Cd group, the NO content was significantly decreased ($P < 0.05$) in the Cd-Se-met group.

Apoptosis-Related Gene Analysis

To understand the effect of Cd on hepatocellular apoptosis in poultry and the protective effect of Se-met on Cd-induced apoptosis, the mRNA and protein levels of apoptosis-related genes were investigated in the liver of broilers (Fig. 4). Compared with the control group, the mRNA and protein levels of detected apoptosis genes were significantly increased ($P < 0.05$) in the Cd-Se-met group and the Cd group. Compared with the Cd group, the mRNA and protein levels of detected apoptosis genes were significantly decreased ($P < 0.05$) in the Cd-Se-met group. Interestingly, the mRNA and protein levels of Bcl-2 were significantly reduced ($P < 0.05$) in the Cd-Se-met group and the Cd group. Compared with the Cd group, the mRNA and protein levels of Bcl-2 were significantly elevated ($P < 0.05$) in the Cd-Se-met group.

PI3K/AKT Pathway–Related Gene Analysis

To investigate whether the PI3K/AKT pathway plays a role in the inhibition of Cd-induced hepatocyte apoptosis by selenium methionine, the P13K/AKT pathway–related genes were analyzed and are shown in Fig. 5. Compared with the control group, the mRNA and protein levels of PI3K and AKT were significantly inhibited ($P < 0.05$) in the Cd-Se-met group and the Cd group. Compared with the Cd group, the mRNA and protein levels of PI3K and AKT were significantly induced ($P < 0.05$) in the Cd-Se-met group, indicating that the PI3K/AKT pathway plays a significant role in apoptosis of Cd-induced hepatocytes inhibited by Se-met.

Heat Shock Proteins–Related Gene Analysis

The heat shock proteins (HSPs)–related genes were analyzed and shown in Fig. 6. Compared with the control group, the mRNA levels of HSP27, HSP40, HSP60, HSP70, and HSP90 were significantly increased ($P < 0.05$) in the Cd-Se-met group and the Cd group. Compared with the Cd group, the mRNA levels of HSP27, HSP40, HSP60, HSP70, and HSP90 were significantly decreased ($P < 0.05$) in the Cd-Se-met group. For protein level, the expressions of HSP60, HSP70, and HSP90 in the Cd-Se-met group and the Cd group were significantly induced ($P < 0.05$) compared with the control group. Compared with the Cd group, the expressions of HSP60, HSP70, and HSP90 were significantly inhibited ($P < 0.05$) in the Cd-Se-met group.

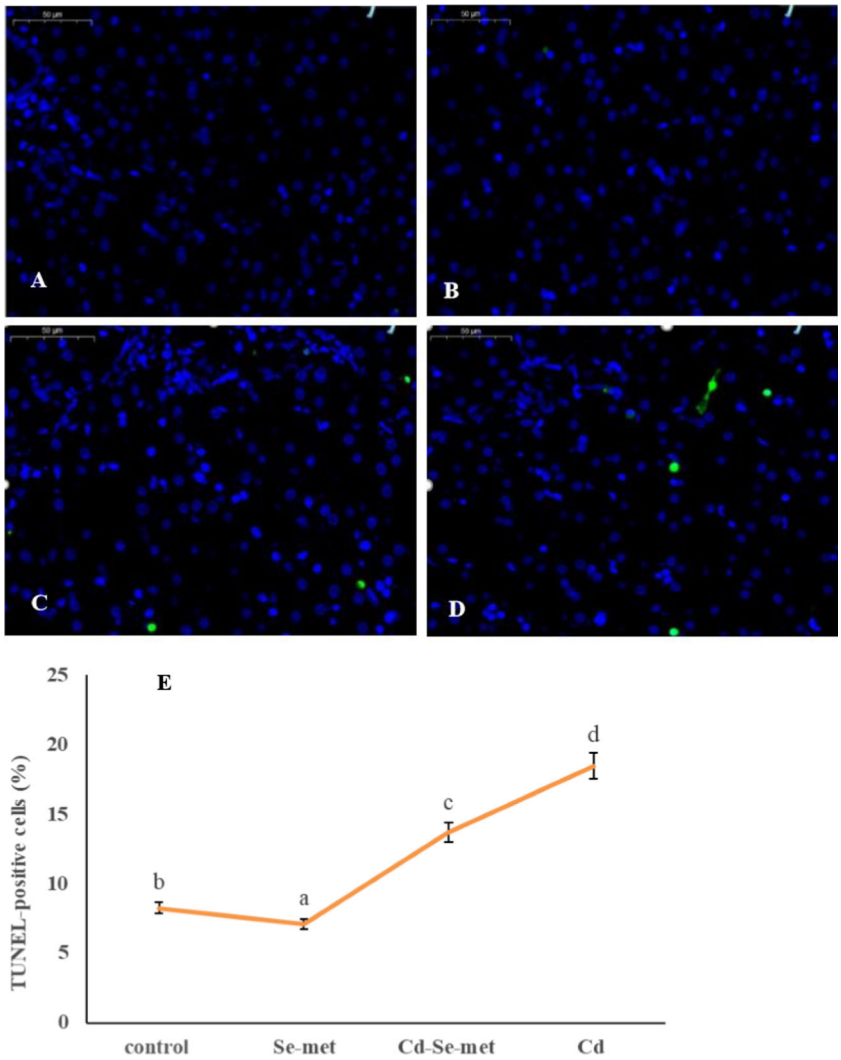

Fig. 1 Apoptosis was measured using TUNEL assay in the liver of broilers. The scale bar represents 50 μm in the upper left corner of all panels. (A) the control group; (B) the Se-met group; (C) the Cd-Se-met group; (D) the Cd group; and (E) the number of TUNEL-positive nuclei. Exposure to Cd remarkably increased the number of TUNEL-positive nuclei (green fluorescence) and the apoptotic index (C). Every figure was showed as × 400 in each group

Discussion

Physiological functions of the liver include detoxification, metabolism, secretion of bile, and immune defense. After the cell is exposed to the poison, its function will be destroyed. It is dependent on homeostatic regulation of cell proliferation, differentiation, and apoptosis [27]. Cd is an environmental pollutant of great concern. Some studies have confirmed that Cd can induce apoptosis [6, 28]. Se is an essential trace element in the body and plays an important role in the antagonistic toxicity of heavy metals. Therefore, we evaluated the antagonistic effects of Se-met on Cd-induced liver toxicity in this study.

Oxidative stress as one of the mechanisms of toxic substances has been widely recognized by researchers [29, 30]. A recent study has found that Cd could alter the natural frequency in oscillations of NADH in mitochondria, thereby contributing to an increase in NADH oxidation rate and disruption of the spatial organization of mitochondria in suspension [31]. Ansari et al. reported that Cd could cause a significant increase ($P < 0.001$) in myocardial MDA content while cardiac GSH level, SOD, and CAT enzyme activities decreased [32]. Our research showed that a decrease in SOD, CAT, and GSH-Px activities and an increase in MDA content in the Cd group. In summary, Cd induced oxidative stress in the liver of chickens. In addition, the changes in the antioxidant values of the Cd-Se-met group were between the Cd group and the control group in our study, suggesting that Se-met can reduce the toxicity of Cd. The reason is that selenium is the main ingredient of some antioxidant enzymes and selenoproteins in the body [9, 10]. Oxidative stress makes the body vulnerable to damage and can enhance the toxic effects of heavy metals [33]. It is not only related to the occurrence and development of many diseases, but also has a close relationship with apoptosis [6].

iNOS is an enzyme that uses NO free radicals to assist macrophages in fighting pathogens in the immune system. It only works after the cells are stimulated and activated, and produces a large amount of NO [34]. NO is an autocrine and paracrine signaling pathway molecule that can diffuse freely

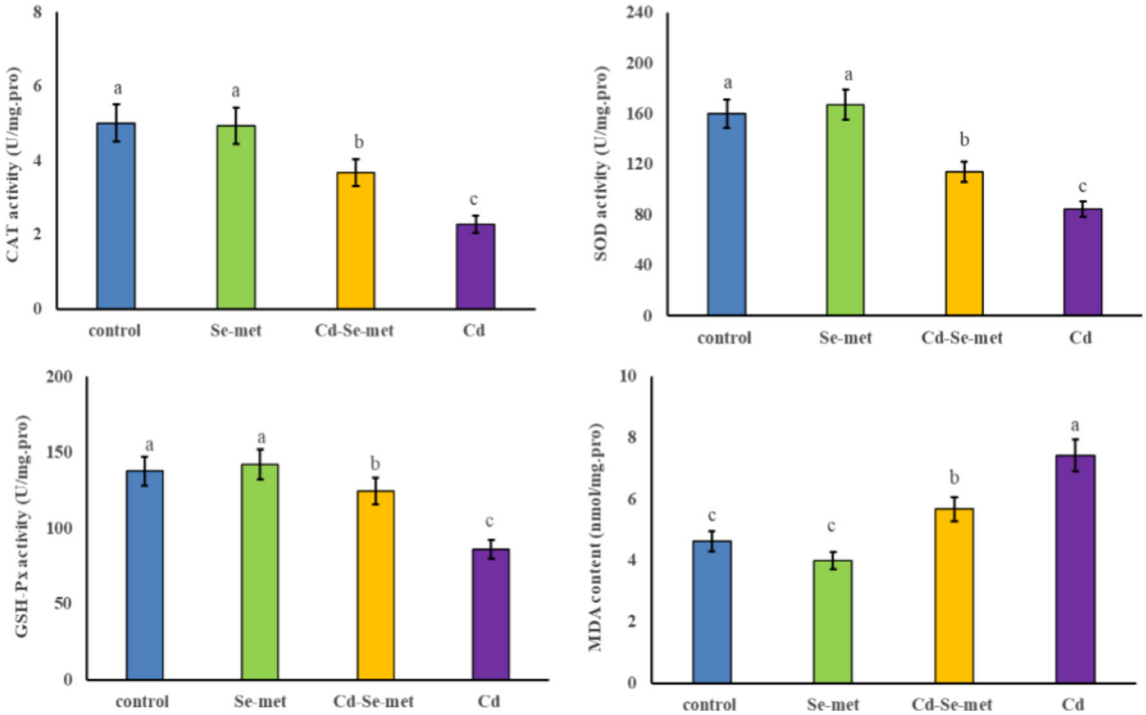

Fig. 2 Effects of Se-met and Cd on antioxidant indicators in the liver of chickens. Each value represents the mean ± SD of 5 individuals. The different lowercase letters at the top of the bars represent significant statistical differences ($P < 0.05$) between different groups. *SOD* Superoxide dismutase, *CAT* Catalase, *GSH-Px* Glutathione peroxidase, *MDA* Malondialdehyde, *Se-met* Selenomethionine, and *Cd* Cadmium

in biofilms. In normal physiological state, the main physiological function of NO is to maintain blood vessel homeostasis and perform signal conduction functions. When cells produce a large amount of NO, NO binds to O_2^- to produce a toxic strong oxidant, which aggravates the damage to the cells. Recent studies showed that lead-induced toxicity was associated with the induction of iNOS and the overproduction of NO in rats [35–37]. Cd exposure led to an increase in iNOS activity and NO overproduction in the chicken pancreas [6]. In our study, the trends of iNOS and NO in the Cd group were consistent with the above reports, indicating that Cd induced stress in chicken hepatocyte. The activation of iNOS can increase NF-κB expression [38]; this mechanism has also been observed in our research. An early study showed that the PI3K/AKT signal pathway was related to the NO synthesis [39]. NO overproduction and the increase of iNOS activity in rat were at least partially mediated by the PI3K/AKT pathway [40]. In addition, the changes in iNOS and NO of the Cd-Se-met group were between the Cd group and the control group in our study, suggesting that Se-met can reduce the toxicity of Cd.

Many environmental pollutants can induce apoptosis, including Cd [7]. In chicken spleen lymphocytes, Cd exposure increased the iNOS and the NO, while overproduction of NO promoted the release of cytochrome C (Cyt C), which in turn induced apoptosis [41]. Bak, Bax, P53, Bcl-2, caspase-3, caspase-9, and Cyt C are important indicators of cell apoptosis [42–44]. In the study of the Tiger frog virus, it was found that

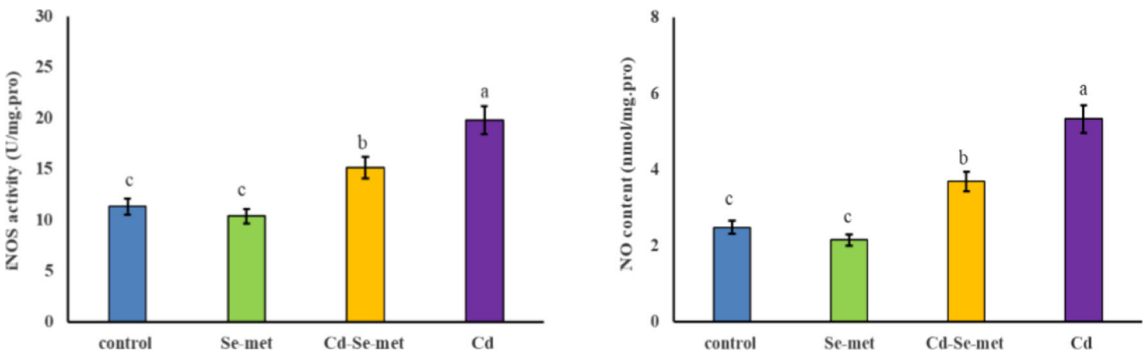

Fig. 3 Effects of Se-met and Cd on NO content and iNOS activity in chicken livers. Each value represents the mean ± SD of 5 individuals. The different lowercase letters at the top of the bars represent significant statistical differences ($P < 0.05$) between different groups. *iNOS* Nitric oxide synthase, *NO* Nitric oxide, *Se-met* Selenomethionine, and *Cd* Cadmium

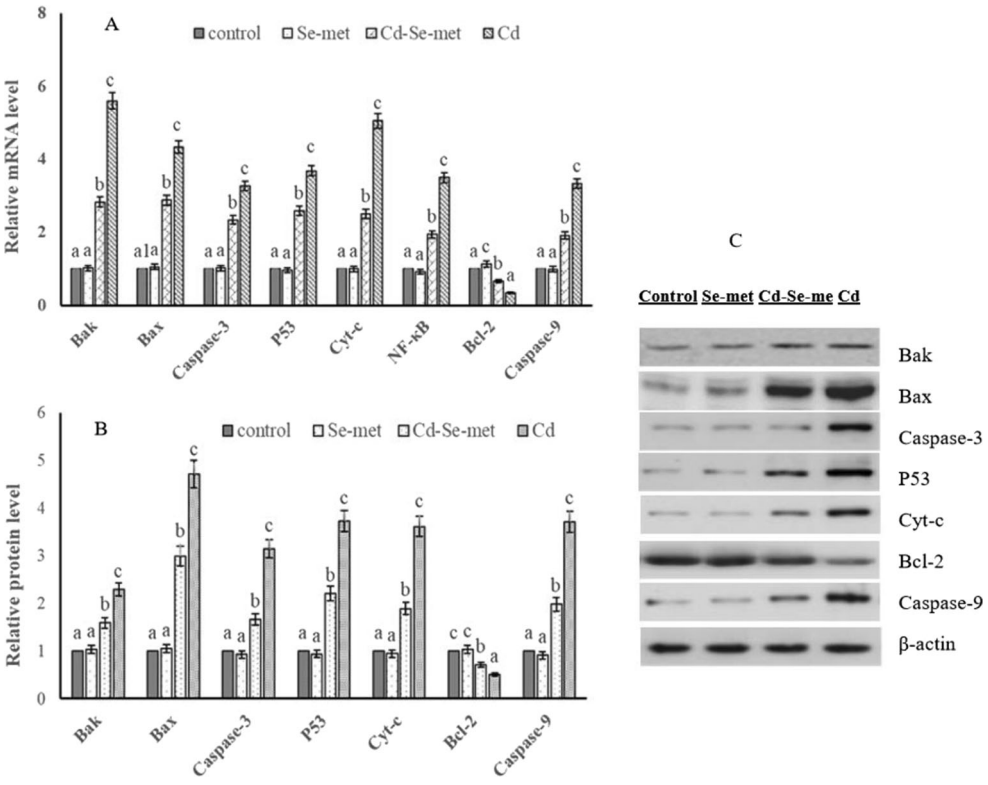

Fig. 4 Effects of Se-met and Cd on the mRNA and protein levels of apoptosis-related genes in the liver of chickens. Each value represents the mean ± SD of 5 individuals. The different lowercase letters at the top of the bars represent significant statistical differences ($P < 0.05$) between different groups. *Bcl-2* BCell lymphoma/leukemia-2, *Bax* BCL2-Associated X Protein, *NF-κB* Neuclear factor kappa B, *Caspase-3* Cysteine-containingaspartate-specificprotease-3, *Caspase-9* Cysteine-containingaspartate-specificprotease-9, *Se-met* Selenomethionine and *Cd* Cadmium

excess Tiger frog virus can inhibit the release of Cyt C and inhibit the activity of caspase-9 and caspase-3, indicating that the Tiger frog virus may play an important role in combating apoptosis [43]. Fisetin inhibited bcl-2 expression and increased the expression of Cyt C, Bax, and BK, which in turn induces apoptosis [44]. In our study, the significant changes in the mRNA and protein levels of detected apoptosis genes (Bak, Bax, P53, Bcl-2, caspase-3, caspase-9, and Cyt C) and

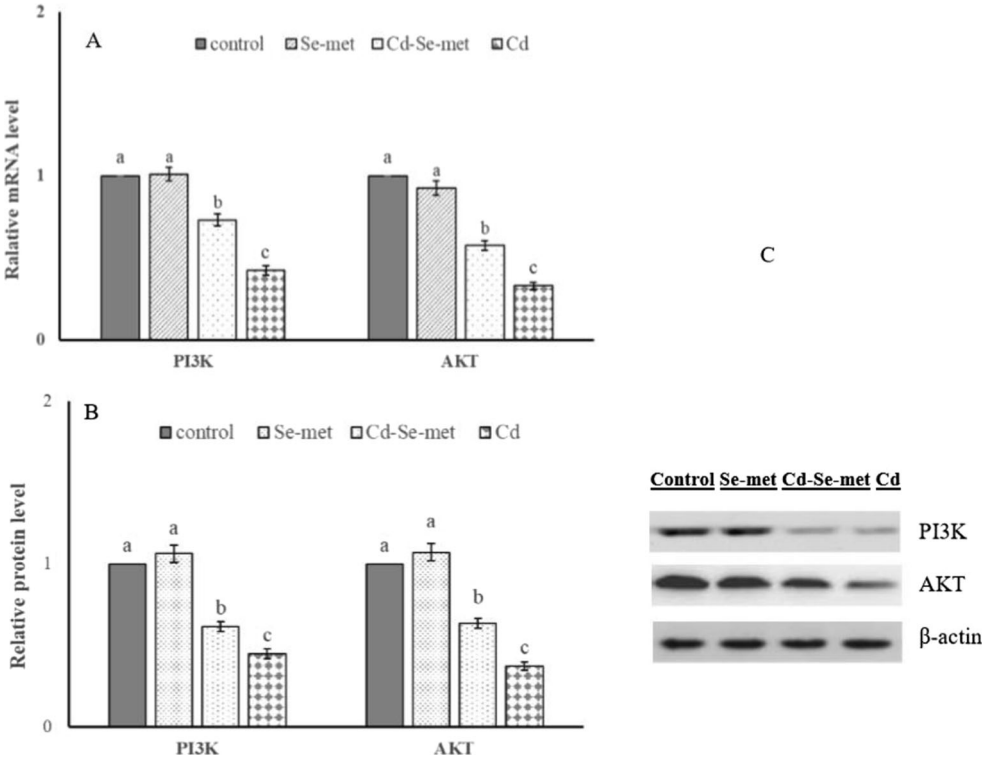

Fig. 5 Effects of Se-met and Cd on the mRNA and protein levels of the P13K/AKT pathway related genes in the liver of chickens. Each value represents the mean ± SD of 5 individuals. The different lowercase letters at the top of the bars represent significant statistical differences ($P < 0.05$) between different groups. *P13K* Phosphatidy linositol-3-kinase, *AKT* Serine/threonine kinase, *Se-met* Selenomethionine and *Cd* Cadmium

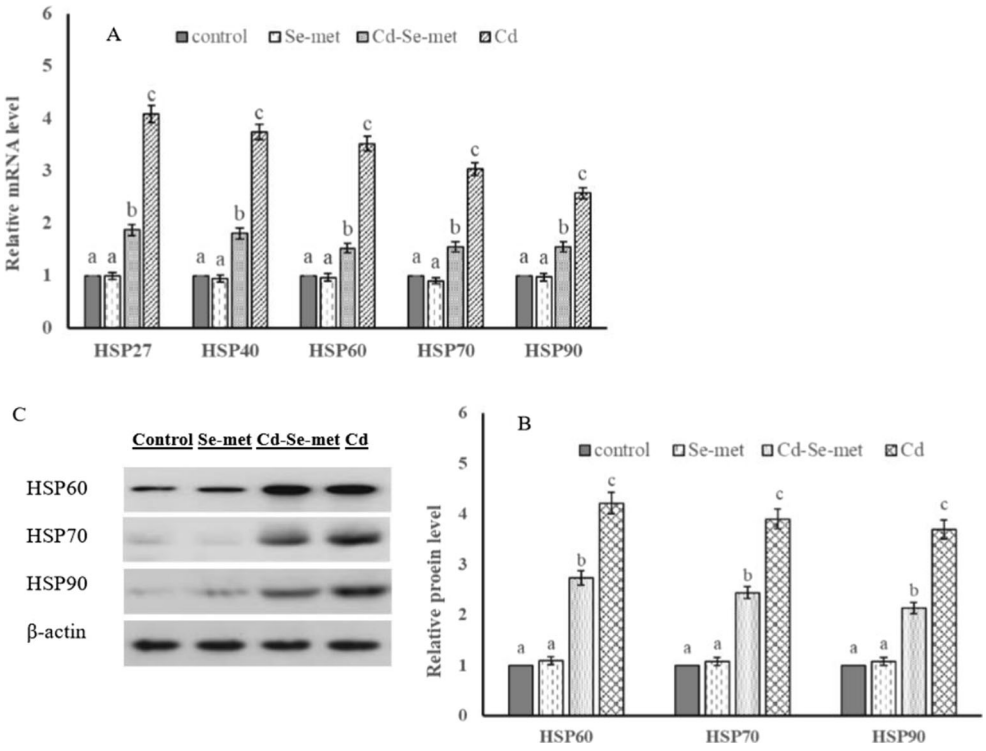

Fig. 6 Effects of Se-met and Cd on the mRNA and protein levels of the HSPs-related genes in chicken livers. Each value represents the mean ± SD of 5 individuals. The different lowercase letters at the top of the bars represent significant statistical differences ($P < 0.05$) between different groups. *HSPs* Heat shock proteins, *Se-met* Selenomethionine and *Cd* Cadmium

the TUNEL result in the Cd group indicated that Cd does induce apoptosis in the liver cells. However, the result of apoptosis genes in the Cd-Se-met group indicated that Se-met can inhibit Cd-induced hepatocyte apoptosis. The PI3K/AKT pathway is closely related to the action mechanism of toxicant. Under external toxic stress, the PI3K/AKT pathway is activated, which in turn induces the occurrence of apoptosis to counter the changes of the internal environment [34, 45]. In our study, the significant changes in the mRNA and protein levels of PI3K and AKT in the Cd group indicated that the PI3K/AKT was an important pathway of Cd-induced apoptosis.

The changes in mRNA and protein expression levels of HSPs are often an important part of the physiological mechanism used by the body to cope with environmental stress, including heavy metals [46, 47]. Acute exposure to Cd can promote high levels of hsp23, hsp24, hsp27, and hsp34 transcript in *Chironomus riparius* larvae [48]. In *Labeo rohita*, exposure to Cd led to the upregulation of HSP47, HSP60, HSP70, HSP78, and HSP90 in kidney and liver tissues, indicating Cd-induced cellular stress. Zhang et al. reported exposure to Cd could increase the mRNA and protein levels of Hsp60, Hsp70, and Hsp90; however, the addition of Se inhibited Cd-induced hepatotoxicity to a certain extent by reducing the expression of HSPs [49]. Our result showed the mRNA levels of HSP27, HSP40, HSP60, HSP70, and HSP90 and the protein levels of HSP60, HSP70, and HSP90 were induced in the liver. The addition of Se-met eased the expression levels of HSP27, HSP40, HSP60, HSP70, and HSP90, indicating that Se-met relieved the environmental stress of the body.

In conclusion, our present study demonstrated that Cd can induce hepatocyte apoptosis and stress in the chickens. Se-met has an ameliorative effect on Cd-induced apoptosis of chicken hepatocyte by regulating PI3K/AKT pathway. Our findings will provide a new insight for better understanding of the detoxification function of Se-met to heavy metals.

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Compliance with Ethical Standards

All experiments were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University.

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

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