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Alleviation Mechanisms of Selenium on Cadmium-Spiked Neutrophil Injury to Chicken

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Abstract To determine the negative effects of cadmium (Cd) exposure and the protective role of selenium (Se) on Cd-spiked neutrophils of chicken, forty-eight 28-day-old Isa Brown male chickens were divided randomly into four groups. Group I (control group) was fed with the basic diet containing 0.2 mg/kg Se. Group II (Se-treated group) was fed with the basic diet supplemented with Na_2SeO_3, and the total Se content was 2 mg/kg. Group III (Se/Cd-treated group) was fed with the basic diet supplemented with Na_2SeO_3; the total Se content was 2 mg/kg and supplemented with 150 mg/kg $CdCl_2$. Group IV (Cd-treated group) was fed with the basic diet supplemented with 150 mg/kg $CdCl_2$. Analyses of inflammatory factors, cytokines, and heat shock protein (Hsp) messenger RNA (mRNA) expression were detected by real-time PCR (RT-PCR). Additionally, we evaluated the phagocytic rate of neutrophils in peripheral blood. First, we observed that Cd significantly induced the mRNA expression levels of inflammatory factors NF-κB, iNOS, COX-2, and TNF-α, while Se/Cd treatment reduced their mRNA expression, although these expression levels remained higher than that of the control group. In addition, the mRNA expression levels of cytokines (IL-2, IL-4, and IL-10) for the Se-treated group exhibited significant differences between the Se/Cd-treated group and the Cd-treated group. Furthermore, the mRNA expression levels of Hsps demonstrated that the Se/Cd-treated group and the Cd-treated group were significantly higher ($P < 0.05$) than the control group and the Se-treated group. These results demonstrated that Se presented partial protection on Cd-spiked neutrophils of chicken with Hsps being involved in the process of the Cd-spiked toxic effects in chicken peripheral blood neutrophils.

Keywords Cadmium · Selenium · Neutrophils · Cytokines · Heat shock protein · Chicken

Introduction

Cadmium (Cd) is both highly toxic and omnipresent in natural environments and is frequently used in many industrial applications, including the production of batteries, metal plating, pigments, and plastics, and through these applications, it can be released into the environment and result in damage to physiological functions and the economy. Different aspects of Cd toxicity have been reported in animals, and the accumulation within the body may produce toxic effects in the kidney, liver, lung [1–4], and reproductive organs [5]. Our previous studies have shown Cd-spiked morphological changes and oxidative stress, ion disorder, and apoptosis, suggesting that the toxic effects of Cd are on the chicken splenic lymphocytes [6]. Cd-spiked accumulation was accompanied by a decrease in variables (glutathione, L-glutathione, superoxide dismutase, and glutathione peroxidase) that lead to injury of the kidney [7]. In addition, Cd is an environmental risk factor for destruction of the immune system. Many studies have shown that exposure to Cd can cause immune system injury [6, 8]. Previous study had indicated that Cd exposure seemed to result in a decreased maturation and mobilization of T and B lymphocytes, thereby affecting the immune system [9]. In some works, high Cd

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levels in *Mus musculus* liver and plasma increased the transcription of hepatic genes involved in oxidative stress and immune response and apoptosis of neutrophils [10].

Selenium (Se) is an important nutritional trace element. In animals, the main function of Se includes antioxidant actions [11–14] and immunity enhancement [15]. Zhang et al. reported that chickens fed with a Se-deficient diet exhibited lesions in immune organs and lower serum IL-1β, IL-2, and TNF content [16]. Also, some reports have clarified that Se is of great importance for human health, protecting cells from the harmful effects of free radicals through significantly increasing the activities of antioxidant enzymes. Zhang et al. indicated that oxidative stress inhibited the development of immune organs and finally impaired the immune function of chickens [16]. Beside these certain roles, some studies have shown that Se was considered one of the most efficient elements guarding against Cd-spiked injury [17, 18]. According to our group preliminary study, Se attenuated the Cd-spiked inflammatory reaction in lymphocytes and enhanced the immune function, which was partly by way of a downregulation of cytokine mRNA expression via the suppression of NF-κB activation in chicken splenic lymphocytes [19]. It has been shown that supplementation of low-dose Se resulted in the decrease of Cd deposition in analyzed organs including livers and kidneys of hens [20]. El-Boshy et al. reported that Se had a potential to countermeasure the immunosuppressive as well as hepatic and renal oxidative damage induced by Cd in rats by regulating the expression of inflammatory factors [21].

Neutrophils were isolated from bone marrow stem cells, which are the most abundant leukocyte, and account for approximately 50–70% of the total of peripheral blood leukocytes [22]. Neutrophils play an important role in nonspecific cellular immunity of organisms as the first line of defense against infectious diseases, and this role has been extensively studied in many immune system diseases of chicken [23]. During immune dysfunction, activated neutrophils have a great capacity to release a wide variety of cytokines and chemokines, which can regulate almost every element of the immune system and abnormal migration [24]. However, there is no clear explanation about the effect of neutrophil phagocytosis against heavy metal poison on the immune system. Snella et al. reported that manganese caused the migration of peritoneal neutrophils from guinea pigs at a significantly higher rate than control animals [25]. It has also been reported that Cd had adverse effects on lymphocytes and induced higher expression of the metallothionein gene on lymphocytes [26]. Meanwhile, increasing information has demonstrated the protective effect of Se against toxicity of Cd. Liu et al. demonstrated that the mRNA expression levels of iNOS, COX-2, and TNF-α in the Se/Cd-treated group were significantly reduced compared to the Cd-treated group in chicken splenic lymphocytes [19]. In addition, Xie et al. indicated that through altered levels of lipid peroxidation and catalase (CAT) activity, Se was protective against Cd toxicity in the least killifish *Heterandria formosa* [27].

Although the negative effects of Cd exposure in mechanistic studies are increasing, limited data about the effects of Se and Cd in neutrophils of chicken have been reported. For discovering the toxic effects of Cd on neutrophils and the antagonistic effect of Se to Cd, we detected the phagocytic rate of neutrophils, inflammatory factors, cytokines, and heat shock protein (Hsp) mRNA expression levels in chicken peripheral blood.

Materials and Methods

Chemicals

Neutrophil isolation extraction kit was purchased from Tianjin Haoyang Biological Products Co., Ltd. TRIzol reagent was purchased from Invitrogen, America. Oligo dT primers and Superscript II reverse transcriptase were provided by Promega, China. The mRNA reverse transcription kit was provided by Thermo Fisher Scientific Inc. Wright's dye was purchased by Beijing Reagan Biotechnology Co., Ltd. *Staphylococcus* was provided by the Northeast Agricultural University pharmacology laboratory.

Preparation of Animals

Forty-eight 28-day-old Isa Brown male chickens were divided randomly into four groups (n = 10 per group). The remaining two chickens in each group were standby for any unexpected condition. Birds were maintained in the Laboratory Animal Center, College of Veterinary Medicine, Northeast Agricultural University, China. The animal room was maintained at 18–26 °C, and the birds were kept under a 16-h light/8-h dark cycle and given free access to standard food and water for 12 weeks. According to the LC_{50} of Cd (218.44 mg/kg diet $CdCl_2$), the exposure doses of Cd (150 mg/kg diet $CdCl_2$) were used in this study. In regard to Se doses, we added and supplied 2 mg/kg Se (supernutritional Se but not Se toxicity) with Na_2SeO_3 supplement. Group I (control group) was fed with the basic diet containing 0.2 mg/kg Se. Group II (Se-treated group) was fed with the basic diet supplemented with Na_2SeO_3; the total Se content was 2 mg/kg. Group III (Se/Cd-treated group) was fed with the basic diet supplemented with Na_2SeO_3; the total Se content was 2 mg/kg and supplemented with 150 mg/kg $CdCl_2$. Group IV (Cd-treated group) was fed with the basic diet supplemented with 150 mg/kg $CdCl_2$. All of the procedures used in this experiment were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University.

In the trial period, no mortality was recorded in all groups. Peripheral blood was quickly taken on the 84th day of the experiment, and then, neutrophils were separated using kits according to the manufacturer's instructions (TBD, China) and stored at −80 °C, in order to isolate the RNA. The other

parts of the peripheral blood were used to make the blood smear for detecting the phagocytic rate.

Phagocytic Rate Testing

Two milliliters of heparin anticoagulant was added to 1 mL of staphylococcus culture and incubated at 37 °C for 10 min after mixing. Twenty microliters of the mixture was placed on a glass slide to prepare blood smears, with seven to eight drops of Wright's dye added to the slide followed by waiting for 3 min; after that, equal amounts of PBS were mixed for 10 min and rinsed with water. The neutrophil phagocytosis rate was detected by microscopic examination observation.

Extraction of Peripheral Blood Neutrophils

Blood was mixed 1:1 with PBS until uniformity was reached, and an equal quantity was added on the surface of neutrophils for liquid separation. This showed four layers of cells from top to bottom in the centrifuge tube, and we collected the second layer and the third layer. They were put in a test tube with equal quantity of PBS and centrifuged for 10 min at 1000 rpm after being thoroughly incorporated, and the supernatant was abandoned; then, an equal quantity of lymphocyte separation medium was added slowly after being mixed with PBS at 1:1 and centrifuged for 20 min at 2000 rpm, and the supernatant was abandoned; the added 10 mL PBS was resuspended and centrifuged for 15 min at 2000 rpm, and the supernatant was abandoned. To a part of them, 1 mL TRIzol was added and resuspended and another part of 1 mL PBS was added and resuspended and then stored at −80 °C for RT-PCR.

Real-Time Quantitative PCR

Total RNA was dissociated from the neutrophils using the TRIzol reagent according to the manufacturer's protocol. The concentration and purity of the total RNA were determined spectrophotometrically at 260/280 nm (Gene Quant 1300/100, General Electric Company, USA). First-strand complementary DNA (cDNA) was synthesized from 5 μg of total RNA using oligo dT primers and Superscript II Reverse Transcriptase according to the manufacturer's instructions (Roche, Switzerland). Synthesized cDNA was diluted five times with sterile water and stored at −80 °C before using.

All of the primers (Table 1) were designed by the Premier software (PREMIER Biosoft International, USA) for RT-

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

Gene	Accession no.	Primer (5′ → 3′)	Product size (bp)
β-Actin	L08165	Forward: CACCACAGCCGAGAGAGAAAT	135
		Reverse: TGACCATCAGGGAGTTCATAGC	
Hsp 40	KB376054	Forward: ACATCCAGGCACCCTCTTTG	300
		Reverse: CCCTTGTCATCCGTGCTCTGT	
Hsp 60	NM_001012916	Forward: AGCCAAAGGGCAGAAATG	208
		Reverse: TACAGCAACAACCTGAAGACC	
Hsp 70	GU441537	Forward: TCCTGATGAGGCTGTTGCTT	189
		Reverse: GTCTGGGTTTGTTTGGTGGG	
Hsp 90	NM_001109785	Forward: TCCTGTCCTGGCTTTAGTTT	143
		Reverse: AGGTGGCATCTCCTCGGT	
IL-1β	NM_204524.1	Forward: CAGCAGCCTCAGCGAAGAG	86
		Reverse: CTGTGGTGTGCTCAGAATCCA-3′	
IL-2	AY510091	Forward: GAACCTCAAGAGTCTTACGGGTCTA	111
		Reverse: ACAAAGTTGGTCAGTTCATGGAGA	
IL-4	AJ621249	Forward: GTGCCCACGCTGTGCTTAC	82
		Reverse: AGGAAACCTCTCCCTGGATGTC	
IL-10	NM0010044142	Forward: CGCTGTCACCGCTTCTTCA	88
		Reverse: TCCCGTTCTCATCCATCTTCTC	
IL-17	AY744450	Forward: CATGTTGTCAGCCAGCATTTCT	107
		Reverse: CATCTTTTTGGGTTAGGCATCC	
IFN-γ	NM_205290.1	Forward: CATCTTTTTGGGTTAGGCATCC	158
		Reverse: ACTGGATGGCTGGCTTGG	
NF-κB	NM_205134	Forward: TCAACGCAGGACCTAAAGACAT	162
		Reverse: GCAGATAGCCAAGTTCAGGATG	
iNOS	NM_204961	Forward: CCTGGAGGTCCTGGAAGAGT	82
		Reverse: CCTGGGTTTCAGAAGTGGC	
COX-2	NM_001167718	Forward: TGTCCTTTCACTGCTTTCCAT	84
		Reverse: TTCCATTGCTGTGTTTGAGGT	
TNF-α	NM_204267	Forward: GCCCTTCCTGTAACCAGATG	82
		Reverse: ACACGACAGCCAAGTCAACG	

PCR. Detected via RT-PCR and gene expression levels were performed on a Light Cycler® 480 System (Roche, Basel, Switzerland) using Fast Universal SYBR Green Master Mix (Roche, Basel, Switzerland). The reactions were performed in a 20-μL reaction mixture containing 10 μL of 2× SYBR Green I PCR Master Mix (Roche, Switzerland), 2 μL of either diluted cDNA, 0.4 μL of each primer (10 μM), 0.4 μL of 50× ROX reference dye II, and 6.8 μL of PCR-grade water. The PCR procedure for all of the primers consisted of heating the reaction mixture at 95 °C for 30 s followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 60 °C for 30 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. The relative mRNA abundance was calculated according to the method of ΔΔCT, accounting for gene-specific efficiencies, and was normalized to the mean mRNA expressions of β-actin.

Statistical Analyses

Statistical analyses of all data were performed using the GraphPad Prism (version 5.0, GraphPad Software Inc., San Diego, CA, USA). When a significant value ($P < 0.05$) was obtained by one-way analysis of variance, further analysis was performed. All data showed a normal distribution and passed equal variance testing. Differences between means were assessed using Tukey's honest significant difference test for post hoc multiple comparisons. Quantitative data are shown as the mean ± SD. Differences of $P < 0.05$ were considered to be significant.

Results

Neutrophil Phagocytosis and Phagocytic Rate in the Chicken Peripheral Blood

To determine the dietary effect on the phagocytic function of neutrophils in different groups, we compared the rate of chicken neutrophil phagocytosis. According to the Fig. 1a–d, under oil immersion lens, we observed the peripheral blood smears, with the field of view showing neutrophils with intracellular bacteria. Phagocytic rates of different groups on chicken neutrophils are shown in Fig. 1e. Obviously, in the Se group, neutrophils had a greater number of phagocytic bacteria compared to the control group, which referred to fact that neutrophil phagocytosis was optimal. In addition, in the Cd-treated group, neutrophil phagocytosis was the worst and the number

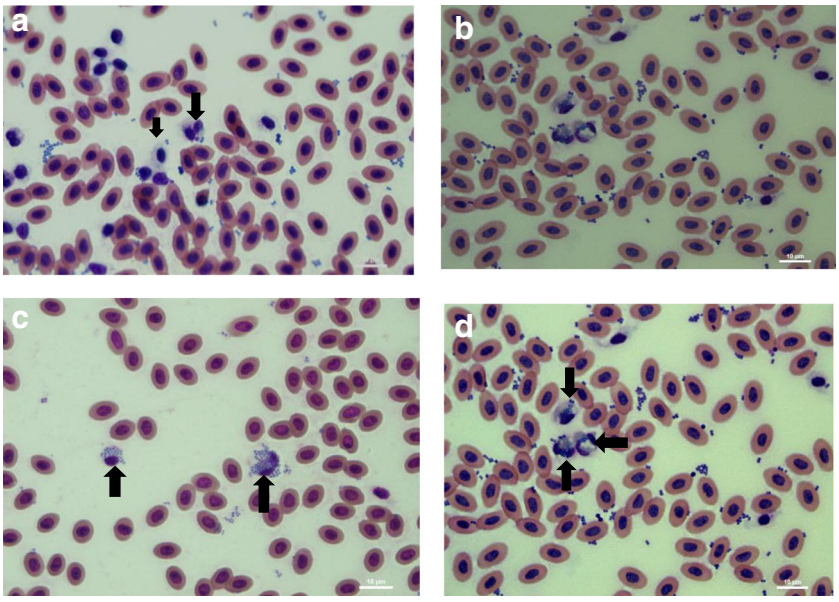

Fig. 1 Phagocytosis and phagocytic rate of different groups on chicken neutrophils. **a** Phagocytosis status from the control group; **b** Phagocytosis status from the Se-treated group; **c** Phagocytosis status from the Se/Cd-treated group; **d** Phagocytosis status from the Cd-treated group. **e** Phagocytic rate of different groups on chicken neutrophils. The *black arrows* in **a**-**d** refer to the intracellular bacteria in peripheral neutrophils

e

Groups	Phagocytic rate(%)
Control group	34.13±2.38[a]
Se-treated group	41.88±1.13[b]
Se/Cd-treated group	34.25±1.75[a]
Cd-treated group	32.38±2.06[a]

of bacteria phagocytized by the neutrophils was the least. Moreover, the number of neutrophils engulfing bacteria of the Se/Cd-treated group was larger than that of the Cd-treated group, which referred to the protective role of Se on the Cd-spiked group.

The mRNA Expression of NF-κB, iNOS, COX-2, and TNF-α from Peripheral Blood Neutrophils

As shown in Fig. 2, the mRNA expression levels of NF-κB, iNOS, COX-2, and TNF-α were examined in peripheral blood neutrophils of chicken. The mRNA expressions of NF-κB, iNOS, COX-2, and TNF-α for the Se/Cd-treated group and the Cd-treated group were significantly higher than those for the control group and the Se-treated group ($P < 0.05$), but there was no significant difference for NF-κB between the Se-treated group and the Se/Cd-treated group. Conversely, mRNA expression levels of these inflammatory factors had significant differences between the Se-treated group and the control group apart from NF-κB ($P < 0.05$). In addition to TNF-α, significant differences existed between the Se/Cd-treated group and the Cd-treated group ($P < 0.05$).

The mRNA Expression of IL-1β, IL-2, IL-4, IL-10, IL-17, and IFN-γ from Peripheral Blood Neutrophils

With regard to cytokines, our results showed that the mRNA levels of IL-2, IL-4, and IL-10 were significantly promoted ($P < 0.05$) in chicken peripheral blood neutrophils of the Se-treated group compared to the control group (Fig. 3). The mRNA expression levels of these genes for the Se-treated group showed significant differences ($P < 0.05$) between the Se/Cd-treated group and the Cd-treated group, of which their values for the Se-treated group were significantly higher ($P < 0.05$) than those for the Se/Cd-treated group apart from IL-1β and IL-17. Moreover, there are no significant differences between the control group and the Cd-treated group for IL-1β, IL-2, and IL-4.

The mRNA Expression of Hsp 40, Hsp 60, Hsp 70, and Hsp 90 from Peripheral Blood Neutrophils

As Fig. 4 illustrates, the mRNA expression levels of Hsps in the Se/Cd-treated group and the Cd-treated group were significantly higher ($P < 0.05$) than in the control group and the Se-treated group. However, the mRNA expression level of Hsp 90 showed no significant difference between the Se-treated group and the control group. Additionally, we also found that mRNA expression levels of these Hsps in the Cd-treated group were significantly higher ($P < 0.05$) than in the Se/Cd-treated group except Hsp 60 and Hsp 90. This result indicated that Cd promoted the mRNA expression of Hsp 40, Hsp 60, Hsp 70, and Hsp 90 mRNA, while Se could alleviate the adverse effect of Cd on the Hsp mRNA expression.

Discussion

Se is an essential trace element in the body with obvious antagonistic Cd effects. Previous studies have already demonstrated the protective effects of Se against subchronic exposure to dietary Cd, and it causes hepatotoxicity, oxidative stress, and apoptosis in chicken liver and kidney [17, 28]. Liu et al. suggested the great importance of Se for chicken

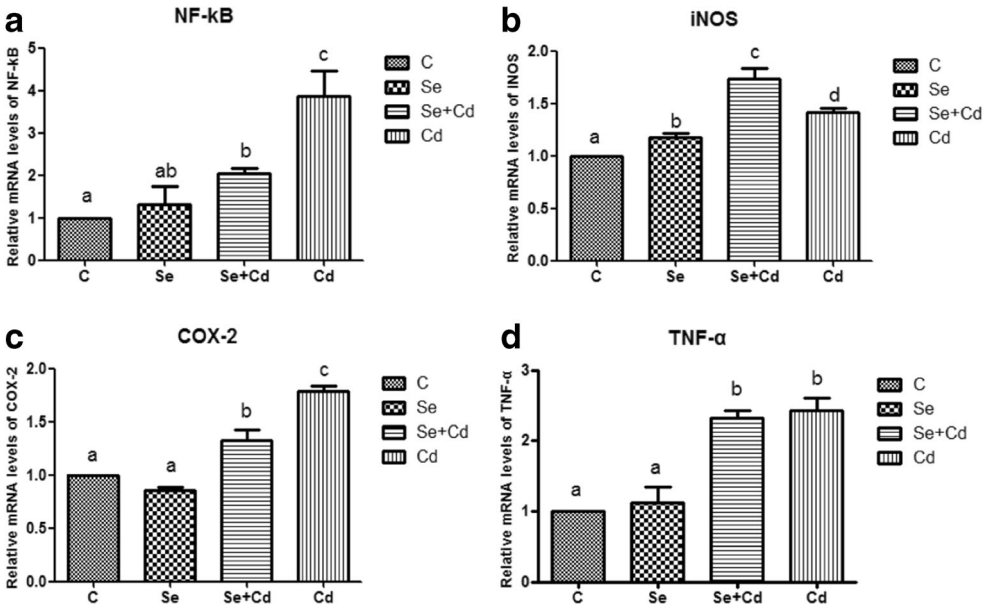

Fig. 2 The mRNA expression of NF-κB, iNOS, COX-2, and TNF-α in peripheral blood neutrophils of different groups. The mRNA levels of NF-κB, iNOS, COX-2, and TNF-α in peripheral blood neutrophils of chicken. Effects of Se on Cd-induced changes in the mRNA levels of NF-κB, iNOS, COX-2, and TNF-α in peripheral blood neutrophils of chicken. The relative mRNA levels from the control group were used as reference values. The *different letters* indicate significant differences ($P < 0.05$) between any two groups. Each value represents the mean ± SD ($n = 10$)

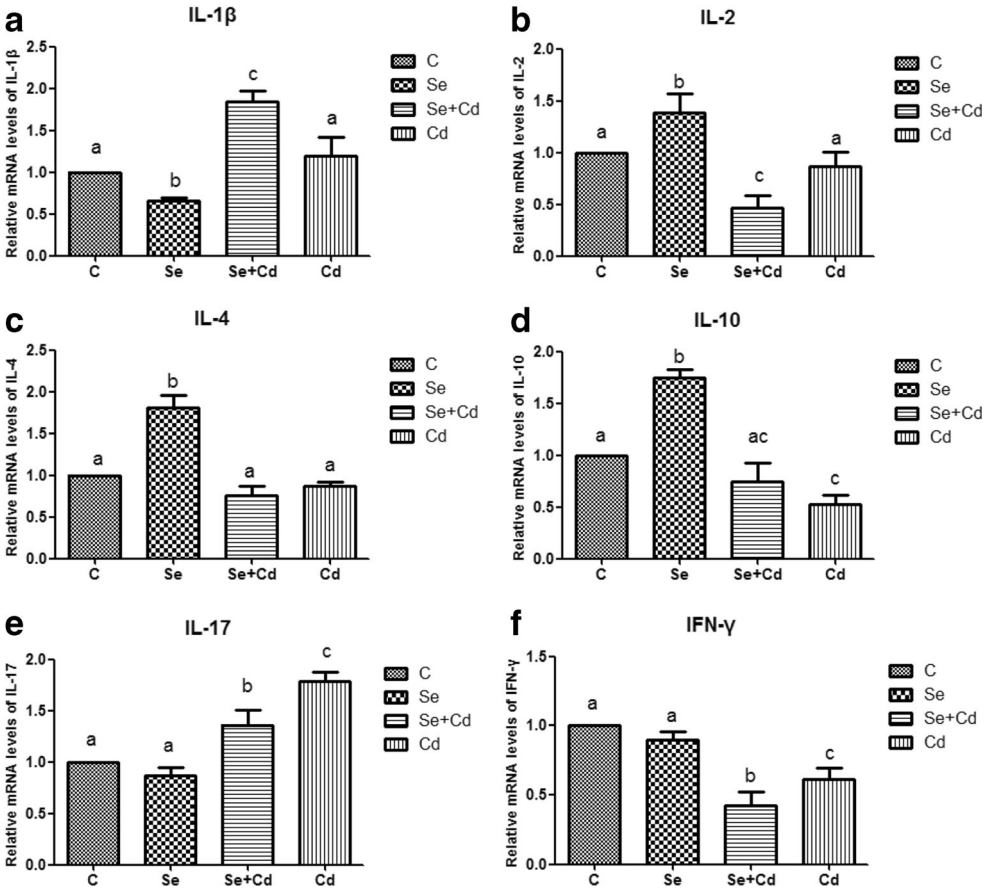

Fig. 3 The mRNA expression of IL-1β, IL-2, IL-4, IL-10, IL-17, and IFN-γ in peripheral blood neutrophils of different groups. The mRNA levels of IL-1β, IL-2, Il-4, IL-10, IL-17, and IFN-γ in peripheral blood neutrophils of chicken. The relative mRNA expression levels from the control group were used as the reference values, and the *different letters* indicate that there were significant differences ($P < 0.05$) between any two groups. Each value represented the mean ± SD ($n = 10$)

immune tissues because Se supplementation during Cd exposure attenuated Cd-induced immune dysfunction [6].

Neutrophils contribute as an essential component to defenses against heavy metal injury through enhancing immune system activity. The numbers of phagocytes and neutrophil phagocytic activity are indicators of nonspecific immune activity. Several metals have been demonstrated to have influence on the phagocytic activity of human neutrophils. With regard to Se and Cd, they can cause a variety of biological effects including alterations of immune responses. To clarify

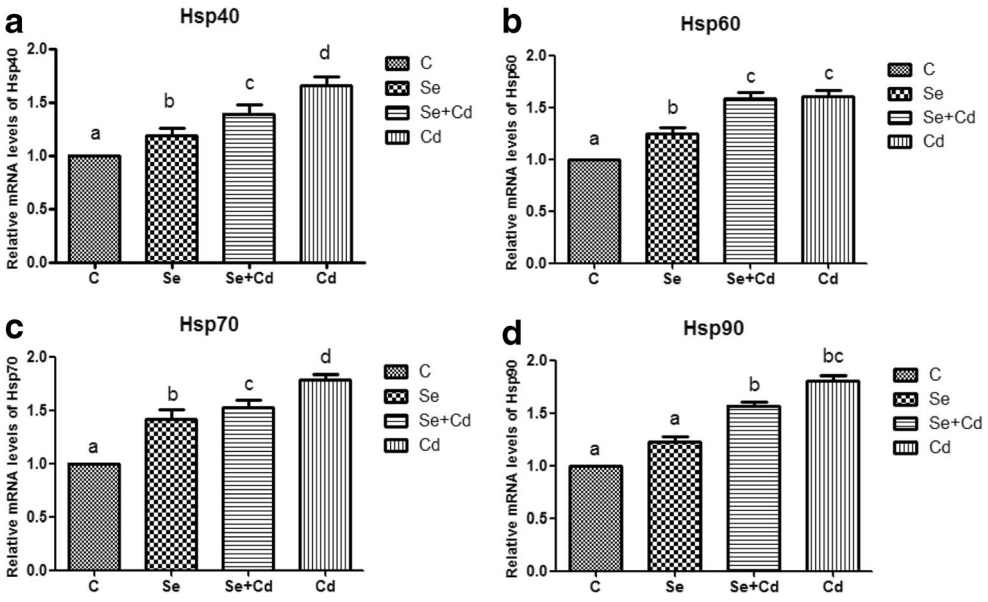

Fig. 4 The mRNA expression of Hsp 40, Hsp 60, Hsp 70, and Hsp 90 in peripheral blood neutrophils of different groups. The mRNA levels of Hsp 40, Hsp 60, Hsp 70, and Hsp 90 in peripheral blood neutrophils of chicken. The relative mRNA expression levels from the control group were used as the reference values, and the *different letters* indicate that there were significant differences ($P < 0.05$) between any two groups. Each value represented the mean ± SD ($n = 10$)

the effects of Se and Cd on the peripheral blood neutrophils of the chicken, we first calculated the phagocytosis of neutrophils under microscope as well as the neutrophil phagocytosis rate of the different groups. Our results revealed that the neutrophil phagocytosis rate of the Se-treated group was the highest and the Cd-treated group was the lowest. Simultaneously, the phagocytosis rate of the Se/Cd-treated group showed slight enhancement compared to the Cd-treated group, which also meant that Se had a potential alleviating role on Cd-spiked peripheral blood neutrophils of chicken. Viñuelas-Zahínos E et al. indicated that the treatment of human neutrophils with Cd did not cause any cellular damage at a concentration of 1×10^{-3} mM for 30 min [29]. The study revealed inconsistent results, which may be, at least in part, due to the different experimental conditions used including different application regimes or varying experimental objects. However, many studies have shown that Cd affected immune cell injuries and Se alleviated this Cd-spiked dysfunction. Liu et al. suggested that Se played a protective role in inflammation induced by Cd via regulating the mRNA expression of inflammatory cytokines in chicken splenic lymphocytes [19]. Similarly, Xu et al. also reported the protective effect of Se against Cd in chicken splenic lymphocytes [8]. Obviously, the results of this study are in accordance with the results of the previously mentioned study. We inferred that the injurious effect of Cd on the phagocytic capacity of neutrophils could be partly changed by Se.

Inflammatory cytokines play a critical role in the inflammatory response induced by various environmental challenges such as exposure to Cd in living organisms, which can also be identified as the predominant mediators of inflammation. This research sought to understand the toxic effect of Cd and the protective role of Se against this toxic effect by determining the mRNA expression levels of inflammatory factors (NF-κB, iNOS, COX-2, and TNF-α) and cytokines (IL-1β, IL-2, IL-4, IL-10, IL-17, and IFN-γ) in the peripheral blood neutrophils of chicken. Our results showed that the mRNA expression levels of inflammatory factors (NF-κB, iNOS, COX-2, and TNF-α) were upregulated in the Cd-treated group and the Se/Cd-treated group compared to the control group. Interestingly, it was higher in the Cd-treated group. Some studies have demonstrated that immune cells' inflammatory response is closely related to the expression of inflammatory cytokines. TNF-α is the most important cytokine, which activates neutrophils and lymphocytes and promotes the synthesis and release of other cytokines [30]. As a pro-inflammatory factor, the main function of iNOS in inflammatory neutrophils induces the production of NO, and NO is a free radical which was synthesized under inflammatory conditions and takes part in immunoregulation [31]. COX-2, another important inflammatory cytokine, not normally expressed in tissues or organs, is stimulated by physiological or pathological condition factors [32]. NF-κB has the ability to participate in regulation of multiple cytokines during different phases of the early immune response and inflammatory response. It has been reported that Cd stimulation caused the expression of ICAM-1 via NF-κB activation in cerebrovascular endothelial cells [33]. Similar to Liu and Cao et al.'s studies [6, 34], in our experiment, the mRNA expression of the inflammatory factors (NF-κB, iNOS, COX-2, and TNF-α) increased due to Cd exposure, but in the Se/Cd-treated group, the mRNA levels of iNOS, COX-2, and TNF-α were significantly reduced compared to the Cd-treated group. Moreover, Låg M et al. have shown that the mRNA expression levels of IL-1β and TNF-α were reduced after exposure to Cd in lung cells from rats [35]. Furthermore, the Se/Cd-treated group showed a significant reversal of the mRNA expression levels of inflammatory cytokines' increases in the Cd-treated group; we identified that this is the first evidence of the effect of Se on mRNA expressions of pro-inflammatory cytokines in the neutrophils of chicken.

With regard to cytokines, our results showed that the mRNA levels of IL-1β, IL-2, IL-4, and IL-10 were significantly promoted in the Se-treated group compared to the control group and the mRNA expression levels of the Se-treated group were significantly higher than the Se/Cd-treated group apart from IL-1β and IL-17. We also found several other studies that have demonstrated that cytokines could interact with each other, thereby regulating the immune system. Like IFN-γ, IL-2, and TNF-α, which were secreted from Th1 cells to enhance the clearance of many intracellular pathogens, there has been a study that has proven that these three cytokines can promote the phagocytic activity of neutrophils. IFN-γ is also important to neutrophil activation because it is necessary for TNF-α to synergize with IFN-γ to induce a variety of neutrophil activating genes, including NOS. It was shown that IFN-γ promoted the level of NO by inducing the release of iNOS [36], then causing an inflammatory response. Moreover, Chen et al. have reported the protective effect of Se against Cd and chicken splenic lymphocytes [37]. El-Boshy M E et al. showed that Se significantly increased the mRNA expression level of IFN-γ, while IL-10 was decreased in rats and Se/Cd treatment significantly improved the elevation of serum IL-1β, TNF-α, and IL-10[21]. To summarize our results, Cd had a toxic effect on chicken neutrophils through inducing inflammation, while Se had a protective role against Cd toxicity.

Hsp is a kind of protective protein produced under adverse conditions that has important roles in the immune defenses of organism [38]. To evaluate the effect of Se and Cd on the mRNA expression of Hsps (Hsp 40, Hsp 60, Hsp 70, and Hsp 90) on neutrophils of chicken peripheral blood, we separated neutrophils from cocks' peripheral blood that received either Se, Cd, or Se/Cd in their diets for 84 days and determined the mRNA expression levels of Hsps. Our results showed that the heavy metal Cd could induce the mRNA expression of Hsps, and this status was slightly reduced by Se but was still higher than the control group. Many studies have shown that the synthesis of Hsps under stress conditions

is one of the first physiological events that protect cells from consequent injury [39, 40]. Cao et al. have reported that the mRNA expressions of Hsp 60, Hsp 70, and Hsp 90 were significantly enhanced in livers of duck, which were exposed to molybdenum and/or Cd [34]. It has also been demonstrated that the mRNA expression levels of Hsp 60 and Hsp 90 in *Labeo rohita* after Cd exposure were significantly upregulated [41]. Brunt JJ et al. have shown that Cd exposure promoted Hsp accumulation in A6 kidney epithelial cells of *Xenopus laevis* [42]. In addition, we detected that the mRNA expression levels of Hsps in the Se/Cd-treated group were reduced compared to those in the Cd-treated group in our study. Chen et al. illustrated that treatment of chicken splenic lymphocytes with Se in combination with Cd promoted the mRNA expression of Hsps, which was reduced by Cd treatment [37]. Quite notably, these results were consistent with our previously mentioned studies.

In summary, Cd treatment decreased the phagocytic rate of neutrophils in peripheral blood. Cd induced the mRNA expression levels of inflammatory factors (NF-κB, iNOS, COX-2, and TNF-α), while the mRNA expression levels of cytokines (IL-2, IL-4, and IL-10 and IFN-γ) were lower than the control group; the mRNA expression level of IL-10 was higher due to Cd. In addition, Cd treatment enhanced the mRNA expression levels of Hsps. However, Se exhibited antagonistic roles against the Cd-spiked inflammatory factors and Hsp mRNA expression levels in chicken peripheral blood neutrophils. We suggested that Hsps were involved in the process of the Cd-spiked toxic effect in chicken peripheral blood neutrophils.

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Compliance with Ethical Standards The Institutional Animal Care and Use Committee of Northeast Agricultural University approved all procedures used in this experiment.

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

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