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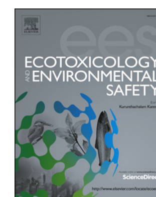

# The protective role of selenium against cadmium-induced hepatotoxicity in laying hens: Expression of Hsps and inflammation-related genes and modulation of elements homeostasis

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ABSTRACT

The purpose of this study was to examine the potential role of high selenium (Se) diets in alleviating chronic cadmium (Cd) hepatic toxicity in laying hens. In the present study, 128 healthy 31-week-old laying hens were fed a diet supplemented with Se ($Na_2SeO_3$, 2 mg/kg), Cd ($CdCl_2$, 150 mg/kg), or both Se and Cd (150 mg/kg of $CdCl_2$ and 2 mg/kg of $Na_2SeO_3$) for 90 days. The expression levels of heat shock proteins (Hsps, including Hsp60, Hsp70 and Hsp90) and inflammation-related factors, including nuclear factor-kappa B p50 (NF-κB), cyclooxygenase-2 (COX-2), prostaglandin E synthases (PTGES), interleukin 1-beta (IL-1β), and tumor necrosis factor-α (TNF-α) were investigated. The concentrations of 28 elements were also determined. The results indicated that Cd treatment significantly increased the mRNA and protein expression levels of Hsps and significantly improved the expression of inflammation-related genes. Moreover, Cd addition to the diets resulted in disturbances in the systemic balance of 13 elements, leading to decrease in the concentrations of Cr, Mn, Sr, Ba, and Hg and increase in Li, B, Ca, Ti, Fe, Cu, Mo, and Cd concentrations. Treatment with Se significantly alleviated Cd-induced hepatic toxicity, as evidenced by a reduction in Hsp60, Hsp70, Hsp90, NF-κB, COX-2, PTGES, TNF-α, and IL-1β expression. Additionally, Se and Cd co-treatment alleviated the changes in Li, B, Ca, Fe, Ti, Cu, Mo, Cd, Cr, Se, Sr, Ba, and Hg concentrations, which was in contrast to that upon Cd induction. The study indicated that Se could help against the negative effects of Cd and may be related to the alleviation of Cd-induced Hsps stress and the inflammatory responses along with modulating the element homeostasis.

## 1. Introduction

Cadmium (Cd), an inorganic heavy metal abundant in the earth's crust, is one of the most well-known environmental toxicants to humans and animals (Nordberg, 2009). The presence of Cd in the soil and animal feed can exceed the maximum permitted limits, leading to a serious problem in some areas (Li et al., 2010; Wang et al., 2015; Zhang et al., 2012). Cadmium is absorbed from the gastrointestinal tract and primarily accumulates in the chicken liver, which can result in hepatotoxicity with pathological damage, lipid peroxidation, and the metabolism disorders of energy and elements (Liu et al., 2015a; Marettova et al., 2015; Muller, 1986; Rikans and Yamano, 2000; Tzirogiannis et al., 2003).

As a defense system to protect bodies from various environment stressors (such as pathogen infection, inflammation, heat or cold shock, heavy metal pollution, etc.), heat shock proteins (Hsps) are synthesized rapidly after exposure to stressors (Sreedhar and Csermely, 2004). Cadmium treatment increased the expression level of Hsp70 in blue mussel, zebra mussel and Chironomus tentans (Lee et al., 2006; Radlowska and Pempkowiak, 2002; Singer et al., 2005), and increased of Hsp90 in pacific oyster and gilthead sea bream (Choi et al., 2008; Garcia-Santos et al., 2011). In addition, the mRNA expression of Hsp60, Hsp70, and Hsp90 were significantly increased in duck livers from dietary molybdenum or/and Cd treated groups where compared to those from the control (Cao et al., 2016a). Also, heavy metals can induce inflammation, and the stress response pathway is enhanced by inflammatory factors such as nuclear factor-kappa B (NF-κB), cyclooxygenase-2 (COX-2), interleukin 1-beta (IL-1β), and tumor necrosis factor-α (TNF-α) (Gupta et al., 2010). Cadmium has the potential to induce immune-toxicity and inflammation in various animal models (Dan et al., 2000; Demenesku et al., 2014; Zhao et al., 2006). For example, in male mice orally administered with several doses of Cd for 7 or 21 days, the mRNA levels of TNF-α, IL-1, IL-6, and interferon γ (IFN-γ) significantly increased in the liver (Liu et al., 2015b). Moreover, it

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was reported that Cd exposure disturbed the metabolism of essential metals, such as calcium (Ca), magnesium (Mg), zinc (Zn), copper (Cu), chromium (Cr) and selenium (Se) (Martelli et al., 2006; Noel et al., 2004). Element homeostasis is necessary for the efficient enzymatic reactions, signal transduction, antioxidant activity, inflammation regulation and other biological processes (Sun et al., 2012). The liver is considered one of the primary tissues for analyzing the stress status and inflammatory response. However, to date, studies about the mechanisms of Cd induced hepatic damage in laying hens are limited.

Selenium (Se) is an essential nutrient for living organisms, known to play a vital role in the antioxidant defense system, and protecting bodies from oxidative and other stress (Yao et al., 2013a). Selenium has been reported to interact with Cd, a majority of studies examining the mechanisms of alleviating Cd hepatic toxicity by Se were conducted for assessing their role in hematological disturbances, oxidative stress, histological changes, or apoptosis (Al-Waeli et al., 2013; Jihen et al., 2008; Li et al., 2013; Liu et al., 2015a; Newairy et al., 2007). However, the association between Cd and Se and the mechanisms by which Se protects against Cd-induced hepatotoxicity in poultry still needs to be explored. Therefore, the present study was conducted to shed some light as to how Se protects Cd-induced hepatotoxicity. The expression levels of Hsps and inflammatory cytokines were determined, and the concentrations of 28 elements were determined in the liver of laying hens that were fed diets supplemented with either Se, or Cd, or both.

## 2. Materials and methods

### 2.1. Hens, diets and experimental protocol

Details of the laying hens and diets used in the study have been described previously (Zhang et al., 2016a, 2017a). Briefly, Hy-line Brown laying hens were collected from a commercial farm at 18 weeks of age. Chickens were fed under the identical standard management until the experiment was started (31 weeks of age). A total of 128 laying hens were randomly divided into four treated groups such that each group had 32 individuals. The basic diet contained 0.2 mg/kg Se and 0.08 mg/kg Cd. The control group was fed the basic diet. The Se treated, Cd treated and Se + Cd treated groups were fed with basic diet supplemented with 2 mg/kg of $Na_2SeO_3$ (total Se content of 1.1 mg/kg), 150 mg/kg of $CdCl_2$ (total Cd content of 92.1 mg/kg), or 150 mg/kg of $CdCl_2$ + 2 mg/kg of $Na_2SeO_3$ (total Se content of 1.1 mg/kg and total Cd content of 92.1 mg/kg), respectively. To make the Se and Cd content in the experimental groups was consistent and the diets were homogeneity, the perfect mixing was accomplished for feed preparation. The composition of basic diet was shown in Supplementary Table S1. The concentrations of 28 minerals in the basic diet and water are described in our previous report (Zhang et al., 2017a) and shown in Supplementary Table S2. All hens were given feed and clean water ad libitum. At the end of the experiment (day 90), hens were euthanized by cervical dislocation. Liver samples were frozen and stored at − 80 °C for RNA isolation and determination of element concentrations. Blood was collected from heart of each hen and centrifuged at 3000 rpm for 10 min to obtain the serum. Serum samples were stored at − 80 °C for assays. All experiments were approved by and conducted according to the guidelines of the Institutional Animal Care and Use Committee of Northeast Agriculture University.

### 2.2. Determination of biochemical parameters

Blood samples used to study changes in serum enzymes are considered to be biochemical indicators of hepatic enzymes. The activities alanine transaminase (ALT), aspartate aminotransferase (AST) and glutamyltranspeptidase (GGT) were measured using commercial detection kits (30 type biochemistry reagent kits, Jiangsu SINNOWA Medical Technology Company, China) by a biochemical auto-analyzer (DS-301 Auto Chem Analyzer, Jiangsu SINNOWA Medical Technology Company, China).

### 2.3. Histopathological examination

Livers from experimental hens were fixed in 10% buffered formalin and embedded in paraffin. Thin sections (5 µm) of each tissue were sliced and stained with hematoxylin and eosin (H&E) for observation (Li et al., 2013).

### 2.4. Reverse-transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of mRNA levels

Primers for amplification of genes Hsp60, Hsp70, Hsp90, NF-κB, COX-2, PTGES, TNF-α, IL-1β, and two reference genes β-actin and glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) were designed using Primer Premier Software 5.0 (PREMIER Biosoft International, CA, USA). The primer sequences are listed in supplementary Table S3. RNA extraction, quality control, RT-qPCR procedure, and relative mRNA quantification were as described in previous report (Liu et al., 2015c). Total RNA was extracted using RNAiso plus reagent (TaKaRa, Dalian, China) according to the manufacturer's instructions. The RNA concentrations in each sample were measured at 260 nm using Nanodrop2000 (Thermo, Waltham, USA) and the purity of total RNA extracted was determined using Nanodrop2000 as the 260 nm/280 nm ratio with expected values between 1.8 and 2.0. The integrity of RNA was determined by RNA electrophoresis on 1% (w/v) agarose gels. The reverse transcription (RT) reactions were performed using a PrimeScript™ RT reagent kit with gDNA Eraser (Takara) according to the manufacturer's instructions. Quantitative PCRs were performed on the LightCycler® 480 system (Roche Applied Science, Shanghai, China) in a final volume of 10 µL using the Roche Fast Universal SYBR Green Master kit (Roche, Shanghai, China). The PCR conditions consisted of 95 °C for 30 s followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s. The PCR reactions were performed in duplicate, and the threshold cycle (Ct) value used in subsequent calculations was the mean of the values from two reactions. The $2^{-\Delta\Delta Ct}$ method was used to calculate the mRNA levels of each target gene (Livak and Schmittgen, 2001). The housekeeping gene GAPDH and β-actin genes were used as internal control for normalization of the results.

### 2.5. Western blot analysis

Protein extracts from livers were subjected to SDS-PAGE under reducing conditions on 12% gels. The separated proteins were then transferred on nitrocellulose membranes using a tank transfer for 2 h at 200 mA in Tris–glycine buffer containing 20% methanol. Membranes were blocked with 5% skim milk at 37 °C, 50 rpm for 2 h. These were then incubated overnight with diluted rabbit antibodies against anti-chicken Hsp60, Hsp70, and Hsp90 polyclonal antibodies (1:1000, made by our lab) and diluted primary antibodies against NF-κB p50, COX-2, and PTGES (1:500, Santa Cruz Biotechnologies, CA, USA). To verify equal loading of samples, membranes were also incubated with the β-actin or the GAPDH antibody (1:1000, Beyotime, China). Bound primary antibodies were then detected by a horseradish peroxidase conjugated Coat Anti-rabbit IgG (1:10,000, Beijing Biosynthesis Biotechnology Co., LTD., China). The signal was measured by enhanced chemiluminescence detection reagents (Applygen Technologies Inc., Beijing, China). Protein bands were visualized by a ChampChemi imaging system (Beijing Sage Creation Science Co. Ltd., Beijing, China).

### 2.6. Measurement of serum TNF-a and IL-1β concentration

Levels of inflammation-related cytokines, TNF-α and IL-1β were measured using commercially available chicken enzyme linked immunosorbent assay kits (Cusabio Biotech Company, Wuhan, China). The procedure followed was as recommended by the manufacturer.

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