Cadmium-induced oviductal magnum injury in laying hens via impairing Nrf2-mediated antioxidant defense and promoting endoplasmic reticulum stress, mitochondrial dysfunction and inflammation

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Research Article

Keywords: cadmium, magnum, Nrf2 signaling, endoplasmic reticulum stress, mitochondrial dysfunction, laying hens

Posted Date: June 15th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1739941/v1

Abstract

This study aims to evaluate the toxic impacts of Cd on the oviductal magnum, which is the main site for the egg-white protein synthesis and secretion. Three hundred and eighty four healthy 38-week-old hens were randomly divided into four treatments. Layers in 4 groups were fed a basal diet (control) and a basal diet with Cd at the level of 15, 30 and 60 mg/kg for 9 weeks. Results showed that Cd could dose-dependently deposited in the magnum tissue and a positive correlation occurred between the accumulation of Cd in the magnum and Cd content in egg whites. When compared with the control group, 60 mg/kg Cd exposure decreased the gene expression of ovomucin (OVM), ovalbumin (OVAL), lysozyme (LYZ) and orosomucoid (ORM1), but increased the gene expression of avidin (AVD) obviously ($P < 0.05$). The level of GSH and the activities of T-SOD and CAT were decreased significantly, while the MDA level was increased obviously in layers treated with 60 mg/kg Cd ($P < 0.05$). The gene and protein levels of Nrf2 and its downstream target molecules, including HO-1, SOD1, SOD2 and SOD3 were significantly down-regulated in 60 mg/kg Cd group ($P < 0.05$). While, the protein level of Keap1 was up-regulated obviously ($P < 0.05$). Besides, Cd exposure induced endoplasmic reticulum stress, mitochondrial dysfunction, disruption of mitochondria-associated endoplasmic reticulum membranes (MAMs), and inflammation accompanied by a significant up-regulation of the gene expressions of Grp78, CHOP, ATF4, ATF6 IRE1α, Grp94, PINK1, Parkin, FUNDC1, GRP75, VDAC1, MCU, MFF, autophagy-related proteins Bnip3, LC3I and LC3II, NF-κB and inflammatory factors IL1β and TNFα, and a significant down-regulation of the gene expressions of MFN1, MFN2 and PGC1α ($P < 0.05$). These results suggested that Cd-induced chicken's magnum toxicity is closely related to inhibition of Nrf2 signaling, ER stress, mitochondrial dysfunction, and disruption of MAMs coupling.

Introduction

Cadmium (Cd) has been recognized as a safety risk to human and animal health. Due to its long half-life, even exposure to low doses of Cd can cause severe degeneration of tissues and organs [1, 2]. With the development of industry and agriculture, Cd pollution in feed and its toxic effects on animals have attracted more and more attention. The main sources of Cd pollution in feed are soil, water, unreasonable processing and utilization of mineral element additives. Cd can accumulate in various organs and tissues of animal body, such as liver [3], ovary [2, 4], uterus [1], kidney and bone [5]. In poultry, the oviduct is an important reproductive organ for the secretion of egg whites and eggshells. Our previous studies have shown that Cd accumulates in the oviduct of laying hens and causes oxidative damage to the eggshell gland, disordered expression of eggshell secretion-related genes, and decreased eggshell quality [1]. In addition, many studies have demonstrated that Cd treatment significantly reduces egg white quality, but the specific mechanism is unclear.

Cd usually leads to excessive accumulation of reactive oxygen species (ROS), which disrupts the free radical scavenging system and eventually induces oxidative stress [6]. Oxidative stress will further induce multiple types of damage through different pathways [7]. The main free radical scavenging system is the enzymatic reaction system in the body, including superoxide dismutase (SOD), catalase (CAT),

peroxidase (POD), etc. The transcription factor NF-E2-related factor 2 (Nrf2) is an important factor regulating cellular oxidative stress and is also a central regulator of maintaining intracellular redox homeostasis. It mainly controls the redox state by regulating cellular oxidative stress-inducing gene clusters [8]. Targeted factors regulated by the Nrf2 signaling pathway, such as heme oxygenase-1 (HO-1), perredoxin MSP23, NAD(P)H quinone dehydrogenase 1 (NQO1), and glutathione S-transferase (GST), play key roles in the adaptive response to oxidative stress [9].

Mitochondria are one of the key intracellular targets of different stressors, including Cd, and one of the main organelles for ROS generation [10]. Cd exposure structurally and functionally compromises the integrity of animal mitochondria. Zhang et al. (2020) showed that Cd treatment induced excessive mitochondrial fission in chicken kidney cells, leading to mitochondrial dysfunction and imbalance of mitochondrial dynamics [11]. The endoplasmic reticulum (ER), a key organelle responsible for cell survival and normal function, is the primary site for synthesis, folding, assembly and modification of most secreted and transmembrane proteins [12]. The disruption of ER homeostasis by a variety of environmental stimuli has been demonstrated to lead to ER stress [13, 14]. Chen et al. (2020) showed that ER stress and mitochondrial dysfunction form a mutually reinforcing interaction [15]. Mitochondrial-associated endoplasmic reticulum membrane (MAMs) is an independent membrane region composed of mitochondria and endoplasmic reticulum that are very close to each other. MAMs not only provide a spatial basis for the interaction between two organelles, but also have become central hubs involved in different cellular life processes, such as calcium signaling, autophagy and apoptosis [16]. In mammalian MAMs, the ER and mitochondria interact with each other through the IP3 receptor (IP3R)-Grp75-voltage-dependent anion-selective channel 1 (VDAC1) trimer complex and the mitochondrial fusion (MFN) proteins MFN1 and MFN2 dimers connect [17]. Recent reports showed that MAMs are involved in cadmium-induced cytotoxicity and autophagy [18, 19].

Egg whites are not only an important source of human nutrition, but also provide essential nutrients for embryonic development [20]. Most ovalbumin (about 90% of total protein) is synthesized and secreted in the oviductal magnum tissue [21]. During the spawning period, the gland cells of the magnum can periodically express a variety of specific proteins, including ovomucin (OVM), ovalbumin (OVAL), lysozyme (LYZ), mucin-like protein-1 (ORM1), anti-biotin (AVD), etc [22], in which OVM plays a key role in the gel properties of egg whites and determines albumen height and Haugh units (HU) [23]. Previous studies have demonstrated that cadmium causes oxidative damage to the reproductive system of laying hens and significantly reduces production performance and egg quality [1, 2]. However, as the main site of egg white synthesis and secretion, the toxic effects of cadmium on oviductal magnum tissue are rarely reported. In the present study, we found that acute Cd exposure caused ER stress and mitochondria dysfunction in the oviductal magnum. We demonstrated for the first time that Nrf2-mediated oxidative stress, ER stress, mitochondrial dysfunction, and disruption of MAMs might contributes to Cd-evoked damage to the oviductal magnum of laying hens.

Materials And Methods

Reagents

CdCl2 • 2.5H2O (purity ≥ 99%) was provided by Sinopharm Chemical Reagent Co., Ltd, Shanghai, China.

Experimental Design, Animals, and Diet

Our previous study has introduced the feeding management of the experimental animals [4]. In short, three hundred and eighty four healthy 38-week-old Hy-Line brown layers with similar body weight and laying rate were randomly divided into four treatments with 6 replicates (16 birds/replicate). Layers in 4 groups were fed a basal diet (control) and a basal diet with Cd (provided as CdCl2·2.5H2O) at the level of 15, 30 and 60 mg/kg for nine weeks. Layers were fed and watered ad libitum throughout the experimental period. The composition of basal diet is presented in Table 1. At the end of experiment, the albumen samples of the eggs (12 eggs per treatment) were collected and stored at -80°C for Cd content determination. Twelve birds in each group were humanely euthanized using cervical dislocation after 12 h of fasting. Immediately removed the oviductal magnum tissue and stored it in liquid nitrogen for Cd residue determination and biochemical and molecular biological analysis. The actual concentrations of Cd in feed, serum and oviductal magnum tissues were detected by graphite furnace atomic absorption spectrometry as decribed by MOHC (2015). The actual Cd concentrations in diets were **0.47 (S.E. 0.01), 15.56 (S.E. 0.18), 30.55 (S.E. 0.11), 60.67 (S.E. 0.15) mg/kg**, respectively.

Table 1

Ingredient compositions and nutrient levels of basal diet for hens[2].

Basal ingredients	Value	Nutrient level	Value
Corn, %	65	Metabolism energy, MJ/kg	2.65
Soybean meal (42.0% CP)	21	Crude protein, %	15.73
Feather meal, %	1	Ether extract, %	6.32
Fish meal, %	1	Lysine, %	0.78
Calcium carbonate, %	7	Methionine, %	0.34
Premix[1], %	5	Cysteine, %	0.32
		Total phosphorus, %	0.61
Total, %	100	Calcium, %	3.45

[1]The premix provided following per kilogram of diet: vitamin A, 7000 IU; vitamin D_3, 2500 IU; vitamin E, 49.5 mg; vitamin K_3, 1 mg; vitamin B_1, 1.5 mg; vitamin B_2, 4 mg; vitamin B_6, 2 mg; vitamin B_{12}, 0.02 mg; niacin, 30 mg; folic acid, 0.55 mg; pantothenic acid, 10 mg; biotin, 0.16 mg; chloride becholine, 400 mg; Cu, 20 mg; Fe, 70 mg; Mn, 100 mg; Zn, 70 mg; I, 0.4 mg; Se, 0.5 mg.

[2]Estimated from Chinese feed database provided with tables of feed composition and nutritive values in China (2015 26th edition).

Oxidative stress indices

About 0.1g of the oviductal magnum tissue was cut and weighted and 9 times ice-cold phosphate buffer solution (PBS) at a ratio of weight (g): volume (ml) = 1:9 was added to make a 10% homogenate. Then, the supernatant was collected for subsequent analysis after centrifugation (3000 rpm, 5 min). The activities of total superoxide dismutase (T-SOD) and catalase (CAT), and the levels of glutathione (GSH) and malondialdehyde (MDA) in the oviductal magnum tissue were detected using the commercially available assay kits procured from Nanjing Jiancheng Bioengineering Institute, China.

Gene Expression Analyses

Total RNA from the oviductal magnum tissue (50–100 mg) was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). PrimeScriptTM RT reagent kit with gDNA eraser (RR047A, Takara) was used to convert total RNA into complementary DNA (cDNA). Quantitative real-time PCR (qRTPCR) was performed with NovoStart SYBR qPCR Supermix Plus (E096-01A, Novoprotein Scientific Inc. China) using the Bio-Rad CFX96 Real-Time PCR System. As shown in Table 2, the primer sequences designed for qRT-PCR were synthesized by Generay Biotech (Shanghai). As Livak and Schmittgen [24] described, the $2^{-\Delta\Delta CT}$ method was used to calculate the relative mRNA level of each gene. The target gene expression levels were normalized to the means of housekeeping gene β-actin.

Table 2
Primer used for quantitative real-time PCR

Target gene	Primer	Primer Sequence (5'-3')	Accession No.
β-actin	Forward	TCCCTGGAGAAGAGCTATGAA	NM_205518.1
	Reverse	CAGGACTCCATACCCAAGAAAG	
Chop	Forward	GCACAGCCCATTTCTGTTTC	XM_015273173.2
	Reverse	TGCCATCCCATTCTGCTAAG	
GRP78	Forward	GTTACTGTGCCAGCCTACTT	NM_205491.1
	Reverse	CCGCTTCGCTTTCTCTACTT	
Nrf2	Forward	CTGCCCAAAACTGCCGTA	NM_205117.1
	Reverse	TCAAATCTTGCTCCAGTTCCA	
NQO1	Forward	CTCCGAGTGCTTTGTCTACG	XM_015874307.1
	Reverse	AATGGCTGGCATCTCAAACC	
HO-1	Forward	GCTGAAGAAAATCGCCCAA	NM_205344.1
	Reverse	ATCTCAAGGGCATTCATTCGG	
SOD1	Forward	TGTGCATGAATTTGGAGACAAC	NM_205064.1
	Reverse	TTGCAGTCACATTGCCGAG	
SOD2	Forward	TGCACTGAAATTCAATGGT	NM_204211.1
	Reverse	GTTTCTCCTTGAAGTTTGCG	
SOD3	Forward	TTTTCTCCTAAAGATGGCAAG	XM_420760.3
	Reverse	CTTCCTGCTCATGGATCACAA	
GST	Forward	GGAAGCCATTTTAATGACAGA	XM_046913335.1
	Reverse	TCCTTTAAAAGCCTGTAGCAGA	
GCLC	Forward	TCTGTAGATGATCGAACGC	XM_419910.4
	Reverse	TCCTTTATTAGGTGCTCGTAG	
GCLM	Forward	GCTGCTAACTCACAATGACC	NM_001007953.1
	Reverse	TGCATGATATAGCCTTTGGAC	
IL1β	Forward	GCCTCTGCTCCCATTTAGTT	XM_015297469.1
	Reverse	CTCACAGTCCTTCGACATCTTC	
IL-6	Forward	CGGTACATACGAGATGGAAACC	XM_015281283.2

Target gene	Primer	Primer Sequence (5'-3')	Accession No.
	Reverse	GATCCGGCAGATGGTGATAAA	
TNFα	Forward	GAGGATTGTGCCCGAACTAAA	AY765397.1
	Reverse	GACAGCCTATGCCAACAAGTA	
NF-κB	Forward	TCCACATCTTTCAGAGCATCAA	XM_015856764.1
	Reverse	CTCCTCAACCTCACTTCCTTAC	
OVM	Forward	GCTGTGTGCTTTACCTCTTTG	XM_040673607.1
	Reverse	GAACTGGGTCCTTCTGTCATC	
OVAL	Forward	GAGAGCAGATCACGGCATATT	MH360742.1
	Reverse	GCTCATCAATTCCTGGGTAGAA	
LYZ	Forward	TACGACACTGGCAACATGAG	KU933268.1
	Reverse	CGGCTGTTGATCTGTAGGATT	
ORM1	Forward	AATGCTGGCCTTCCTTGT	NM_204541.2
	Reverse	GACGTGGATCTTACTGGAGTTC	
AVD	Forward	CCTACATCACAGCCGTAACA	AM779412.1
	Reverse	GCTTTCCAGTCATCACCAATG	

Table 2
Continued

Target gene	Primer	Primer Sequence (5'-3')	Accession No.
MFN1	Forward	GTTGTTGGCGGAGTGATTTG	XM_046923917.1
	Reverse	CTTGGAGTGGCTCTGTATGTT	
MFN2	Forward	GACAGGTTGCCTTGTGAGATAG	XM_040689233.2
	Reverse	CCCATTCTTACCCTGGCATTAG	
PINK1	Forward	AGCAGCGATTCCTTCATCTC	NM_001389481.2
	Reverse	CTTCAGGTCTCTGTGTGCTATC	
Parkin	Forward	GTCCAGCAAAGCATCGTTCA	XM_046914604.1
	Reverse	CAACGATGGAAGGATGCTGG	
Fundc1	Forward	GTTGTGCTGGGTTCTCATTTAC	NM_001276363.2
	Reverse	TGTTCCTCGGCGTTTCTT	
GRP75	Forward	TGTGTCAGCCAAGGACAAAG	NM_001006147.2
	Reverse	GAGGATCCACCAGGATTTCA	
VDAC1	Forward	GCCTGAAGCTGACTTTTGACTCC	XM_046927011.1
	Reverse	GATGTGCTCCCTTTTGTATCCTGT	
MCU	Forward	TTGGCAGAGTGTGAGAGTGG	XM_046920626.1
	Reverse	AATTCCTCGGTCCTCTGCTT	
LC3I	Forward	TTACACCCATATCAGATTCTTG	XM_040688401.2
	Reverse	ATTCCAACCTGTCCCTCA	
LC3II	Forward	AGTGAAGTGTAGCAGGATGA	NM_001031461.2
	Reverse	AAGCCTTGTGAACGAGAT	
MFF	Forward	ACTCAAAGTGGCTCCTCA	XM_040679333.2
	Reverse	CCTGCATAGTTACACTGG	
PGC1α	Forward	TACAGCAATGAGCCTGCCAA	XM_046916274.1
	Reverse	AGGCAATCCATCCTCATCCAC	
ATF4	Forward	TCACCCAATGACAACCCG	NM_204880.3
	Reverse	TCACCTTTGCTGACGCTACC	
ATF6	Forward	CGTCGTCTGAACCACTTACTGA	XM_040677276.2

Target gene	Primer	Primer Sequence (5'-3')	Accession No.
	Reverse	CCTTCTTTCCTAACAGCCACAC	
IRE	Forward	CTACAGGTCGCTCCTCACATC	XM_046927561.1
	Reverse	ATCAGTCCTTCTGCTCCCATCT	
GRP94	Forward	CAAAGACATGCTGAGGCGAGT	NM_204289.2
	Reverse	TCCACCTTTGCATCCAGGTCA	

Western Blot analysis

Approximately 0.5 mg oviduct magnum tissue was taken and homogenized in the lysis buffer for the total protein extraction. BCA protein determination kit obtained from Beyotime (Shanghai, China) was used to measure the concentrations of total protein. Then, equal volumes of SDS-PAGE loading buffer were added to protein samples and heated at 100℃ for 5 minutes to denature the protein for the subsequent WB analysis. Western blot analysis was performed according to the previous description [2]. Simply put, proteins were separated using gradient SDS-PAGE (4–20%, GenScript, catalog no. M00657) and then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore). After 12–16 h of incubation with Nrf2 (dilution 1:1000, catalog no. AF7904, Affinity), Kelch-like ECH-associated protein 1 (Keap1) (dilution 1:1000, catalog no. AF7335, Affinity) and GAPDH (dilution 1:5000, as a loading control) antibodies, the membranes were rinsed three times using TBS buffer (1×) containing 0.1% Tween-20. Then, the blots were incubated with the secondary antibody (HRP-labeled goat anti-rabbit IgG, dilution 1:5000, catalog no. bs-0295G-HRP, Bioss) for two hours at room temperature. ECL reagent kit (Beyotime) was used for the visual detection of proteins. Digital images were captured using ChemiScope 3400 (Clinx Science Instruments, China).

Statistical analysis

The IBM SPSS Statistics 20.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The test data were presented in the form of mean ± standard error (S.E.). Pearson correlation analyses was used to evaluate bivariate correlations. One-way ANOVA followed by Tukey test for post-hoc analyses was used to compare significant difference between groups ($P < 0.05$). Linear regression analysis was used to assess the dose-response relationship of Cd exposure with Cd content in serum and magnum tissue.

Results

Cadmium contents in serum and oviductal magnum tissues

Cd contents in albumen and magnum are showed in Fig. 1. The contents of Cd in albumen and magnum were increased linearly with the increase of dietary Cd exposure. The results of linear regression analysis showed that Cd contents in albumen and magnum are significantly correlated with the Cd contents in diet

(R^2 = 0.9766, linear P = 0.0118; R^2 = 0.9204, linear P = 0.0406). There was a significant positive correlation between the Cd content in albumen and the Cd content in the magnum tissue (R^2 = 0.8387, linear P < 0.01).

Effect of Cd on the expressions of genes related to egg-white protein

Because of Cd treatment significantly decreased the egg-white quality, we evaluated the expressions of the genes related to egg-white protein synthesis and the results were shown in Fig. 2. When compared with the compared with the control group, the mRNA level of OVM gene was up-regulated obviously in the group treatment with 15 mg/kg Cd (P < 0.05). However, when layers were treated with 30 or 60 mg/kg Cd, the expressions of OVM, OVAL, LYZ and ORM1 genes were significantly downregulated (P < 0.05). In contrast, the expression of AVD gene was upregulated obviously in the group treated with 60 mg/kg Cd (P < 0.05).

Effect of Cd on antioxidant parameters in the oviductal magnum of layers

Dietary Cd exposure disrupted the antioxidant status in the oviductal magnum tissues of layers and the results were shown in Fig. 3. When layers treatment with 60 mg/kg Cd, the GSH content and the T-SOD and CAT activities were significantly decreased (P < 0.05). In contrast, the MDA level was increased significantly compared with control group (P < 0.05).

Effect of Cd on the Nrf2 signaling pathway in the oviductal magnum of layers

The results of dietary Cd exposure affecting the Nrf2 signaling pathway are presented in the Fig. 4 and Fig. 5. When compared with the control group, the expressions of NQO1 and HO-1 genes were up-regulated obviously in layers treated with 15 mg/kg Cd and then down-regulated in layers treated with 30 or 60 mg/kg Cd. In layers treated with 30 and 60 mg/kg Cd, the mRNA level of Nrf2 was down-regulated obviously (P < 0.05). Besides, the mRNA levels of Nrf2-dependent genes SOD1, glutamate-cysteine ligase catalytic subunit (GCLC) and glutamate-cysteine ligase modified subunit (GCLM) were significantly upregulated in layers treated with 15 or 30 mg/kg Cd (P < 0.05), while the expressions of SOD1, SOD2 and SOD3 were downregulated in layers treated with 60 mg/kg Cd obviously (P < 0.05). The mRNA level of glutathione S-transferase (GST) was upregulated significantly in 60 mg/kg Cd group (P < 0.05) (Fig. 4). Results from western blot revealed that, when compared with the control group, the expressions of Nrf2 proteins were downregulated in layers treated with 30 and 60 mg/kg Cd obviously (P < 0.05). However, 30 and 60 mg/kg Cd exposure induced a significant upregulation of Keap1 protein, the key repressor of Nrf2 (P < 0.05) (Fig. 5).

Effect of Cd on the inflammatory response in oviductal magnum

As presented in the Fig. 6, the mRNA levels of pro-inflammatory factors (interleukin 6 (IL-6), interleukin 1β (IL-1β) and tumor necrosis factor α (TNFα)), and nuclear factor kappa-B (NF-κB) were performed by qRT-PCR. The 60 mg/kg Cd exposure obviously up-regulated the expressions of IL-1β, TNFα and NF-κB genes ($P < 0.05$). In contrast, the expression of IL-6 gene was downregulated obviously in layers treated with 30 and 60 mg/kg Cd compared with the control group ($P < 0.05$).

Effect of Cd on mitochondrial dysfunction

We evaluated the effect of Cd on mitochondrial function and dynamics and the results were shown in Fig. 7. When compared with the control group, the mRNA levels of mfn1, mfn2 and peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α) were down-regulated significantly in the 60 mg/kg Cd group ($P < 0.05$). The mRNA levels of the PTEN-induced kinase 1 (PINK1), Parkin RBR E3 ubiquitin-protein ligase (Parkin), FUN14 domain containing 1 (FUNDC1), VDAC1, mitochondrial calcium uniporter (MCU), mitochondrial fission factor (MFF), and autophagy-related proteins Bnip3 and LC3 were upregulated significantly in 30 or 60 mg/kg Cd treatment groups compared with the control group ($P < 0.05$).

Effect of Cd on endoplasmic reticulum stress

The genes related to ER-stress were detected and the result was presented in Fig. 8. The expressions of the activating transcription factor 4 (ATF4), activating transcription factor 6 (ATF6), Inositol Requiring Enzyme 1 (IRE1), ER chaperones genes CCAAT-enhancer-binding protein homologous protein (Chop), glucose regulated protein 78 (Grp78) and glucose regulated protein 94 (Grp94) were up-regulated significantly in the group treatment with 60 mg/kg Cd compared with the control group ($P < 0.05$).

Discussion

Cadmium is a bioaccumulative toxic heavy metal element that can accumulate in reproductive organs and cause reproductive system disorders [1, 25]. In the present study, we observed that Cd could dose-dependently deposited in the magnum tissue and a positive correlation occurred between the accumulation of Cd in the magnum and Cd content in egg whites. Previous researches have indicated that environmental Cd exposure was usually associated with chickens' fallopian tube damage and poor egg quality, including poor eggshell and egg white quality [26]. The oviductal magnum epithelial cells of laying hens synthesize and secrete a large amount of egg white proteins in daily cycles, including OVM, OVAL, LYZ, ORM1 and AVD, among which OVM plays a key role in the gel properties of egg white and determines the albumen height and Haugh unit of egg white [23, 27, 28]. We assessed the impacts of dietary Cd on the expressions of major genes involved in egg white protein synthesis in oviductal magnum of laying hens. Results indicated that the expression of OVM gene was up-regulated in 15

mg/kg Cd group, but the expressions of OVM, OVAL, LYZ and ORM1 genes were obviously down-regulated when layers treated with 60 mg/kg Cd. Ovalbumin is the most abundant protein in egg white, accounting for more than 50% of the total egg protein [29]. Lysozyme is a well-known antibacterial protein, which accounts for about 3.4% of the total egg white proteins [30]. In contrast, we found that 60mg/kg Cd exposure obviously increased the expression of AVD gene, which is also a critical egg white antimicrobial protein with a strong ability to bind biotin [31]. However, the increased concentration of egg white avidin may adversely affect the innate immunity of newborn chicks [32]. Therefore, these results indicated that Cd could accumulate in the oviductal magnum of laying hens, which was closely related to the Cd content in egg white. Besides, Cd affects the synthesis of egg white protein by disturbing the expression of egg white-related protein genes in the oviductal magnum, but the specific mechanism needs to be further explored.

Cadmium has been known as a reproductive toxicant, and the induction of oxidative stress is widely considered as one of the major mechanisms by which Cd exerts reproductive toxicity [33]. Cd is implicated in the increase of ROS and the induction of oxidative stress through indirect mechanisms, that is, by affecting the activity of ROS scavengers or depleting GSH [34, 35]. Cd exposure can lead to oxidative damage and apoptosis of hens' shell-gland, kidney, ovary and hepatocytes [1, 2, 3, 4]. In the present study, the concentration of Cd exposure decreased the activities of CAT and T-SOD and the level of GSH, and increased the content of MDA in the magnum tissue. These specific biomarkers are considered to be closely related to oxidative stress responded to Cd exposure [9]. A major function of Nrf2 that has been extensively studied is its role in resistance to oxidative stress [36]. Previous studies have indicated that the Nrf2-knockout mice are more susceptible to chemical toxicity and disease conditions related to oxidative pathology [37, 38]. Nrf2 is considered to be the "master regulator" of the antioxidant reaction, which protects cells from the toxicity of free radical by modulating the expression of genes encoding antioxidants through interacting with the antioxidant response elements (ARE) [39, 40]. Many studies have shown that Nrf2 signaling pathway can attenuate the toxicity of multiple organs induced by Cd, including in the kidney, testicular, liver, etc. [7, 41, 42]. In this study, the transcription or protein expression level of Nrf2 were obviously downregulated in 30 and 60 mg/kg Cd treatment groups. Meanwhile, the expressions of Nrf2 target genes NQO1, HO-1, GCLC and GCLM also showed similar trends. Keap1, as a key endogenous repressor of Nrf2, can control the stability and accumulation of Nrf2 [43, 44]. In this study, the rapid up-regulation of Keap1 protein expression level in the 60 mg/kg Cd group further confirmed the inhibitory effect of Cd on the Nrf2 signaling pathway. Besides, the expressions of Nrf2-activited genes SOD1, SOD2 and SOD3 genes were down-regulated significantly in the 60 mg/kg Cd treatment group. These results suggested that 60 mg/kg Cd exposure induced oxidative stress through inhibiting Keap1-Nrf2-ARE signaling pathway and the activities of antioxidant enzymes in the magnum tissues of laying hens.

Inflammation is a protective response of organism to injury. Many studies have indicated that Cd exposure impaired innate immune parameters, triggered ROS generation and inflammation [1, 3]. Consistently, the current study found that the relatively high-dose Cd exposure upregulated the expressions of TNFα, IL-1β and NF-κB genes obviously in the magnum of laying hens. IL-1 and TNFα

represent the typically pro-inflammatory cytokines that are released rapidly in response to tissue damage or infection [45, 46], which have also been identified as the downstream targets of NF-κB [47]. Studies demonstrated that Nrf2 not only participates in the regulation of oxidative/xenobiotic stress response, but also suppresses the inflammatory response. The decrease in Nrf2 with subsequent increase in oxidative stress markers led to the activation of TGF-β and NF-kB [48]. These results suggested that Cd exposure triggered an inflammatory response in the magnum tissue, and this effect may be mediated by activating NF-κB via inhibiting the Nrf2/HO-1 signaling pathway.

Studies suggest that mitochondrial dysfunction may contribute to reproductive toxicity in response to Cd [49]. Mitochondrial structural damage and mitochondria-mediated apoptosis and autophagy have been reported in Cd-treated chicken liver and kidney [7, 50]. For the genes involved in mitochondria dysfunction, the present study indicated that Cd exposure down-regulated the expressions of Mfn1 and Mfn2, and up-regulated the mRNA level of MFF in the oviductal magnum of laying hens. The formation of excess ROS depends heavily on dysfunctional mitochondria, and it is important to note that mitochondrial ROS formation can result in the opening of the mitochondrial permeability transition pore (mPTP) [51]. During conditions of excess ROS and Ca^{2+} overload, opening the mPTP helps to maintain Ca^{2+} levels in the mitochondrial matrix [52]. However, in dysregulated mPTP, matrix metabolites are released, which is accompanied by depolarization of the mitochondrial membrane potential (MMP) and inhibition of oxidative phosphorylation, ultimately leading to mitochondrial damage [53, 54]. In the present study, we found that dietary Cd exposure upregulated the mRNA levels of mPTP-related proteins, including GRP75, VDAC1 and MCU obviously. Known as a master regulator of mitochondrial biogenesis, PGC-1α plays a role in energy metabolism and mitochondria homeostasis [55]. A reduction in PGC-1α expression appears to active PINK1/Parkin-mediated mitophagy [56]. Studies have shown that the PINK1/Parkin pathway is the primary mechanism involved in mitophagy, which occurs when the balance of mitochondrial division and fusion is upset by Cd [57]. Accordingly, we found that exposure to Cd results in a decrease in PGC1α gene level in magnum while expression of PINK1, Parkin, Bnip3, LC3I and LC3II increases. These results suggested that, after exposure to Cd, the expression of mitochondrial fusion factors was limited, whereas the expression of mitochondrial fission factors was enhanced, which caused an imbalance in mitochondrial dynamics. Mitophagy is triggered by mitochondrial damage mediated by PINK1/Parkin.

In previous studies, ER stress (ERS) was found to induce mitophagy. Autophagy may be thought of as a cyto-protective response to an overload of unfolded or misfolded proteins during ERS [58]. Gao et al. (2020) demonstrated that blocking activation of the ERS response prevented the mitophagy process initiated by plumbum [59]. In the present study, we found that the expressions of ATF4, ATF6, IRE1α, CHOP, GRP78 and GRP94 were increased in the group of 60 mg/kg Cd, indicating that Cd can induce ER stress by activating ATF4, IRE1 and ATF6 signaling pathways in the magnum of laying hens. Several regulatory components link the ERS, unfolded protein response (UPR) and mitochondrial function, of which MAMs play a central role. ATF4 was reported to control expression of the ubiquitin ligase Parkin, a crucial regulator of mitochondria function and dynamics [60]. Phosphorylated MFN2 is a receptor of Parkin, which interacts with Parkin to promote the ubiquitination of mitochondrial proteins [61]. Xin et al.

(2019) indicated that knockdown of Mfn2 led to aggravation of the PERK/ATF4 pathway [62]. Lin et al. (2021) demonstrated that excessive ROS accumulation is a critical activator of ERS, leading to mitochondrial Ca^{2+} accumulation via IP_3R–GRP75–VDAC1 complex, and consequently programmed necrosis [63]. These results suggest that Cd exposure can promote mitophagy by inducing ER stress and disrupting the MAMs coupling.

Conclusion

This study results collectively show that Cd can dose-dependently accumulate in the oviductal magnum. Cd-induced chicken's magnum toxicity is closely related to inhibition of Nrf2 signaling, ER stress, mitochondrial dysfunction, and disruption of MAMs coupling. Our findings provide a new insight highlighting the critical role of oxidative stress, ER stress and mitochondrial dysfunction in Cd affecting egg white protein synthesis in the oviductal magnum of laying hens.

Declarations

Funding

This study was supported by the earmarked fund for PhD research start-up fund of Jiangsu University of Science and Technology (No.1732932002) and the Modern Agro-industry Technology Research System of China (CARS-40-K10).

Competing Interests

The authors declare that they have no conflict of interest.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Mingkun Zhu. The first draft of the manuscript was written by Mingkun Zhu and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

Data will be made available on reasonable request.

Ethics approval

All experimental procedures and humane end points for minimizing suffering was approved by the Animal Care and Welfare Committee and the Scientific Ethical Committee of the Zhejiang University (NO. ZJU2013105002), Hangzhou, China.

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Figures

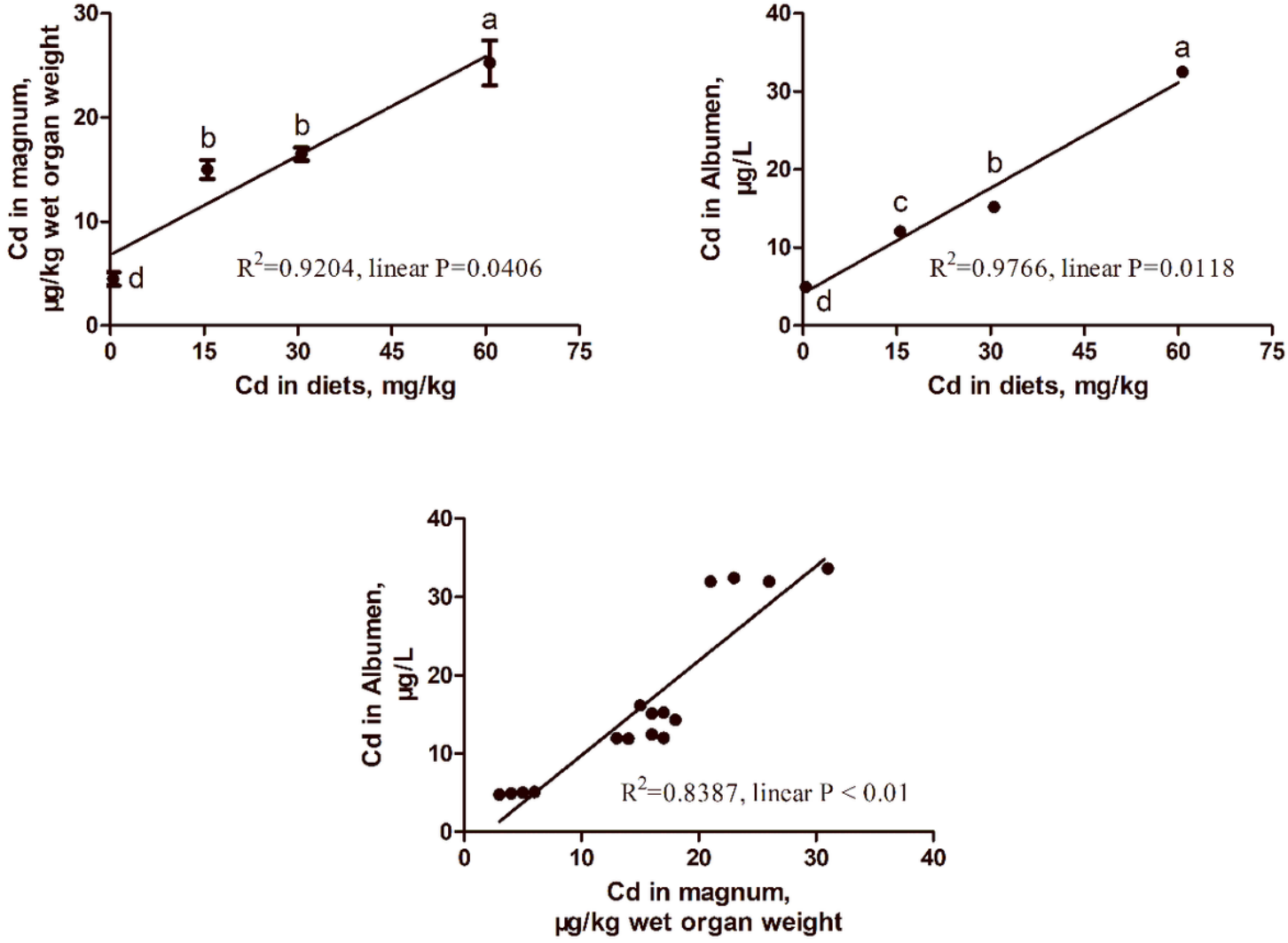

Figure 1

Effects of dietary cadmium contamination on cadmium levels in albumen and magnum of laying hens. Data are presented as mean ± standard error (S.E.) (n = 12). Different letters (a, b, c, d) indicate significant differences between groups ($P < 0.05$). Linear regression analysis was used to assess the dose-response relationship between Cd exposure and Cd contents in albumen and magnum.

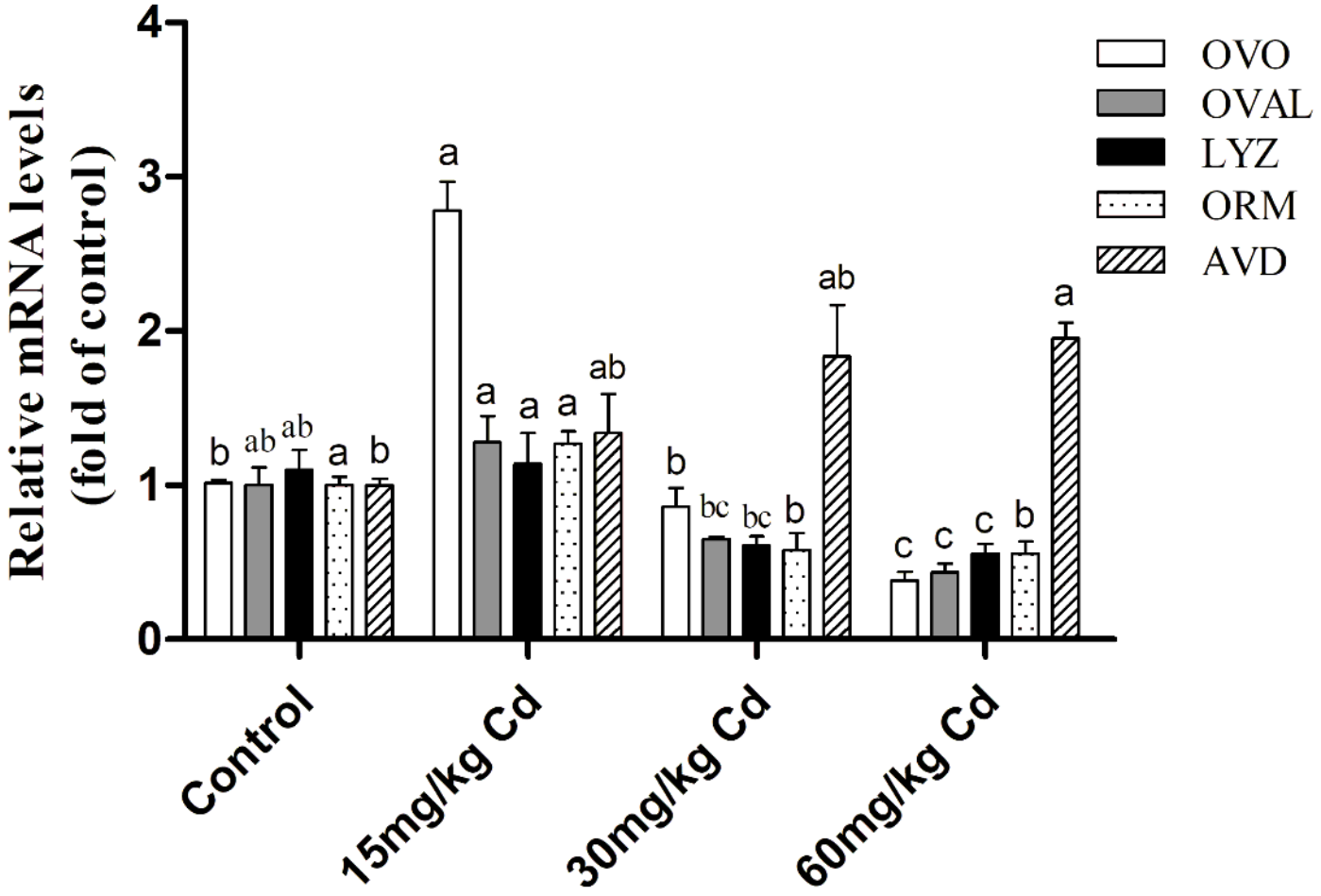

Figure 2

Effects of Cd on the egg white-related gene expressions in the oviductal magnum of laying hens. Different letters (a, b, c) above the histogram indicate significant differences between groups ($P < 0.05$). OVM, ovomucin; OVAL, ovalbumin; LYZ, lysozyme; ORM, mucinoid; AVD, avidin.

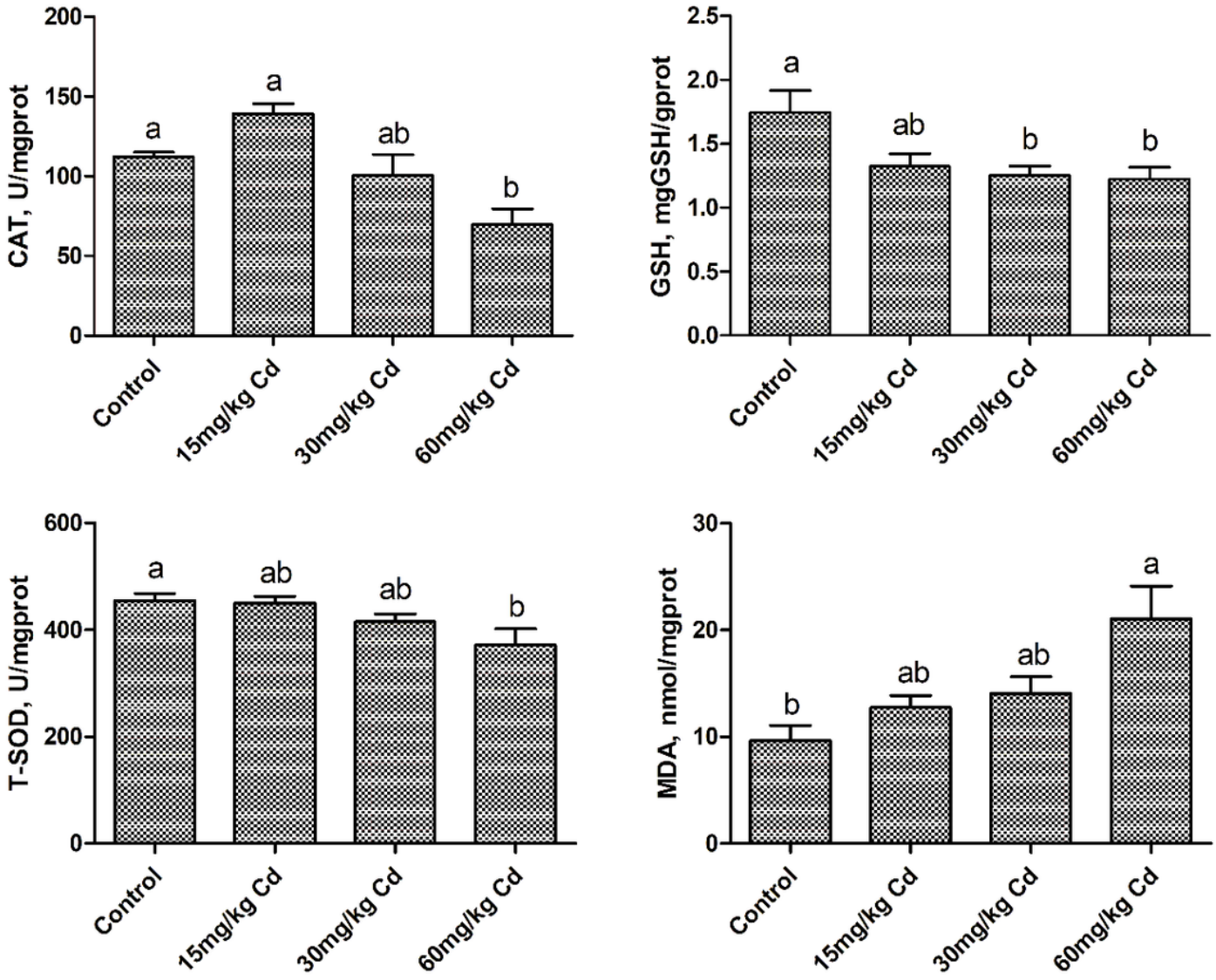

Figure 3

Effect of Cd on the antioxidant ability of the oviductal magnum of laying hens. Data are presented as mean ± standard error (S.E.) (n = 6). Different letters (a, b, c) above the histogram indicate significant differences between groups ($P < 0.05$). GSH, glutathione; T-SOD, total superoxide dismutase; CAT, catalase; MDA, malondialdehyde.

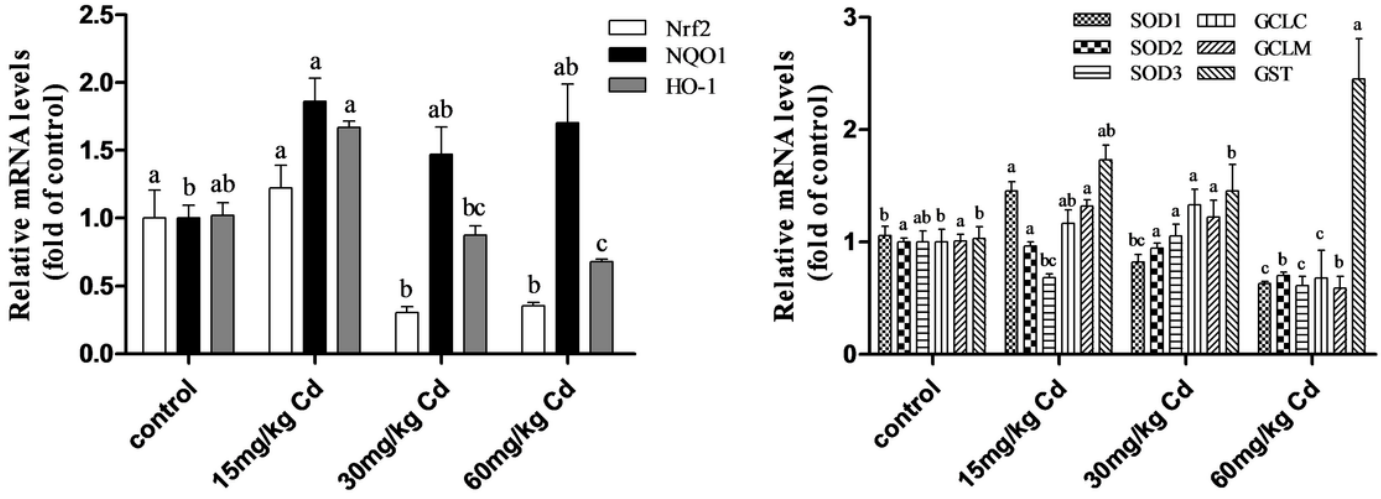

Figure 4

Effects of Cd on the gene expression of (A) Nrf2, NQO1, HO-1, (B) SOD1, SOD2, SOD3, GCLC and GCLM in the oviductal magnum of laying hens. Data are presented as mean ± standard error (S.E.) (n = 6). Different letters (a, b, c, d) above the histogram indicate significant differences between groups ($P < 0.05$).

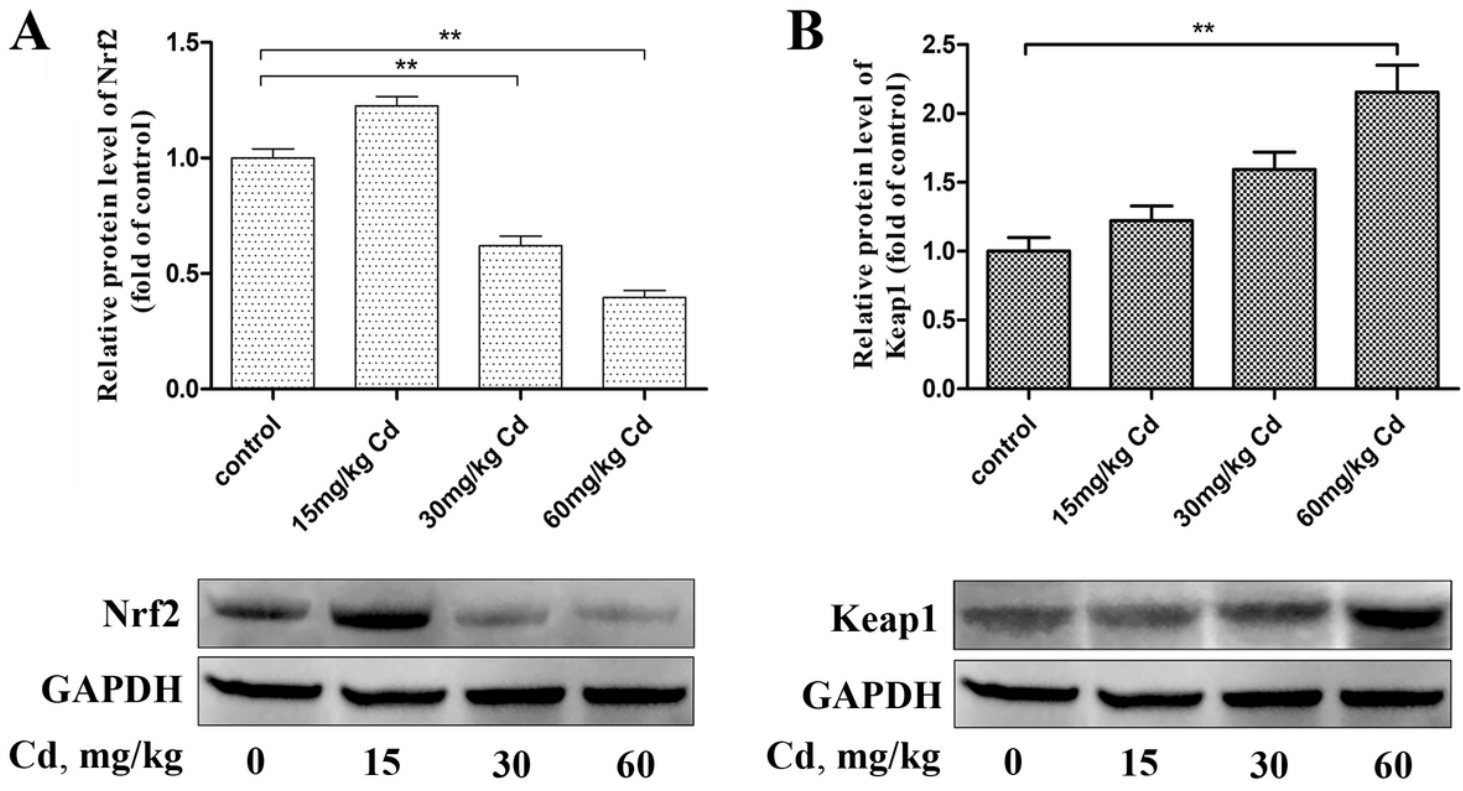

Figure 5

Effects of Cd on the expression levels of Nrf2 and Keap1 proteins in the oviductal magnum of laying hens. (A) Nrf2 protein expression level; (B) Keap1 protein expression level. Data are presented as mean ± standard error (S.E.) (n = 6). $^{**}P < 0.01$ indicates a significant difference compared to the control group.

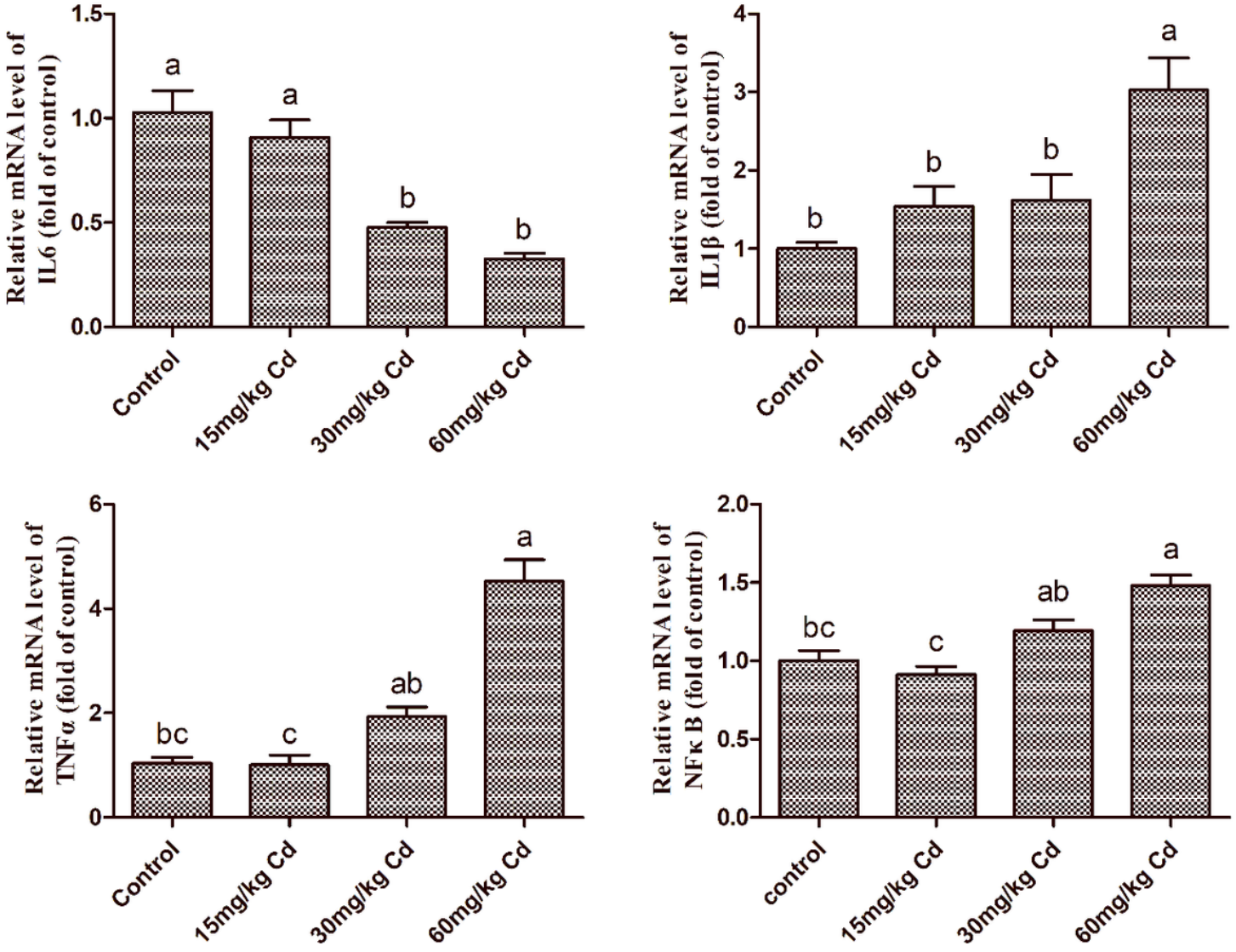

Figure 6

Effects of Cd on the gene expression of inflammatory factors in the oviductal magnum of laying hens. Data are presented as mean ± standard error (S.E.) (n = 6). Different letters (a, b, c) above the histogram indicate significant differences between groups ($P < 0.05$).

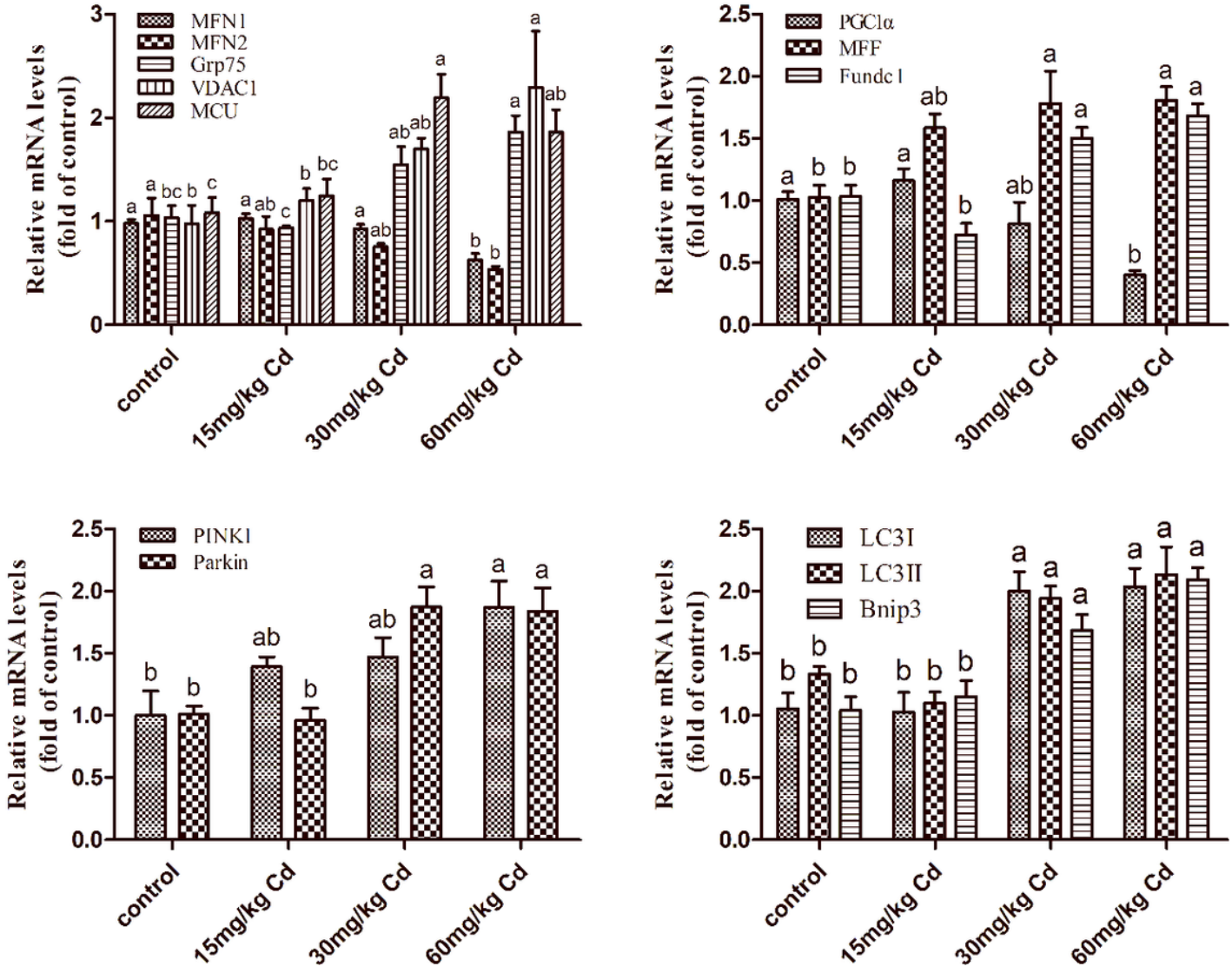

Figure 7

Effects of Cd on mitochondrial homeostasis in the oviductal magnum of laying hens. Data are presented as mean ± standard error (S.E.) (n = 6). Different letters (a, b, c) above the histogram indicate significant differences between groups ($P < 0.05$).

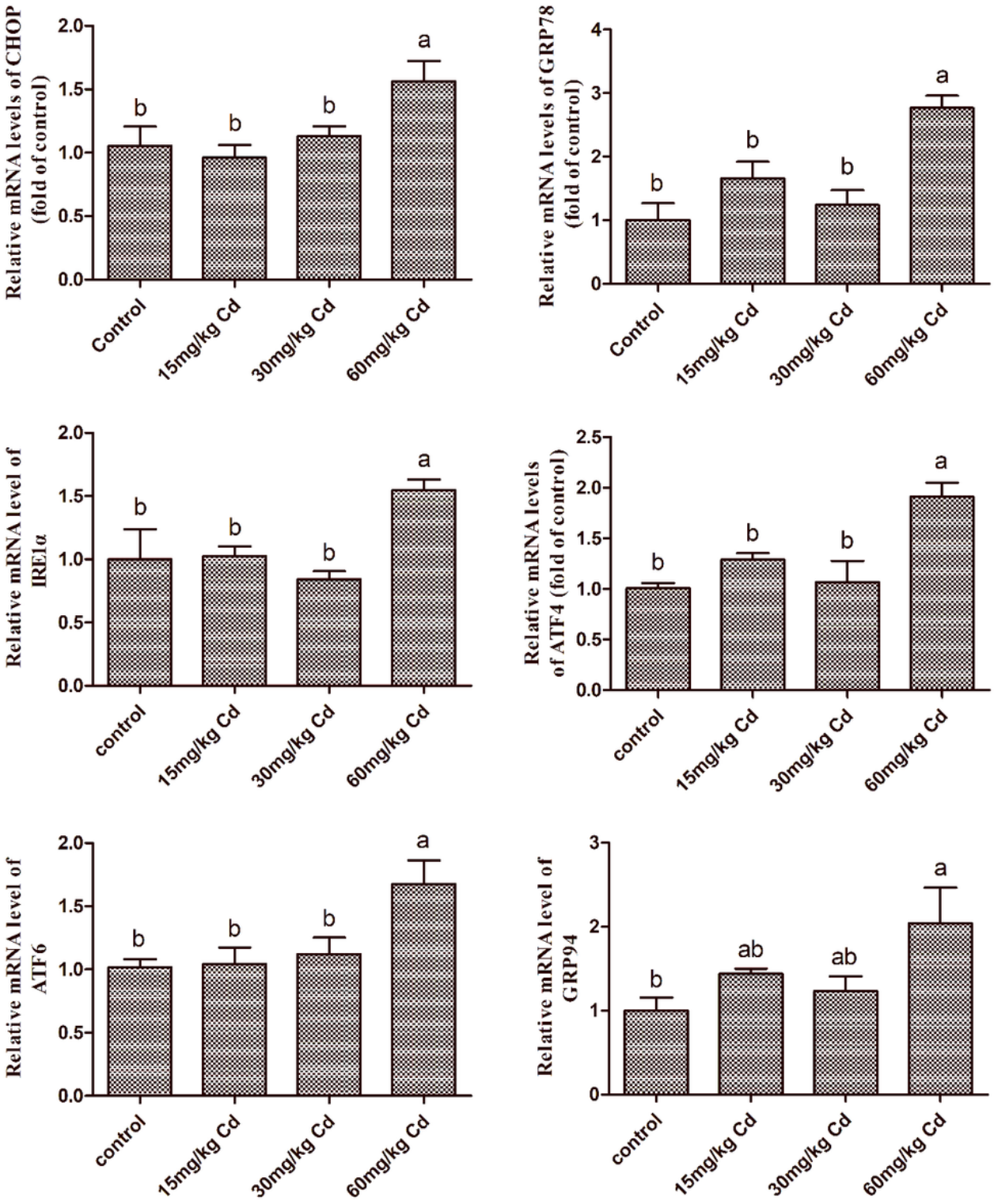

Figure 8

Effects of Cd on ER-stress in the oviductal magnum of laying hens. Data are presented as mean ± standard error (S.E.) (n = 6). Different letters (a, b, c) above the histogram indicate significant differences between groups ($P < 0.05$)