Transcriptomics and transmission ultrastructural examination reveals the nephrotoxicity of cadmium in laying hens

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Received: 28 June 2021 / Accepted: 26 December 2021 / Published online: 31 January 2022
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Abstract
The objective of this study was to reveal the effects of cadmium (Cd) on ultrastructural changes, oxidative stress, and transcriptome expression in the kidneys of laying hens. Seventy-two healthy Hy-Line brown laying hens at 41 weeks old were randomly allocated to four treatment groups with six replicates. The control group received a basal diet without additional Cd incorporation, and the other three treatment groups received diets supplemented with 15, 30, or 60 mg Cd/kg of feed. After 6 weeks of exposure, the results show that administration of 60 mg/kg Cd significantly reduced \( P < 0.05 \) eggshell thickness. With an increase in the Cd concentration in feed, the concentrations of renal Zn and Fe also had changed. Renal histopathology and ultrastructure also showed aggravated damage to glomeruli and renal tubules and the deformation of nuclei and mitochondria in all Cd treatment groups. With an increase in Cd in feed, the activity of glutathione peroxide (GPX) and catalase (CAT) was significantly reduced \( P < 0.05 \), while the activity of total antioxidant capacity (T–AOC) was decreased \( P < 0.05 \) only in the 60 mg/kg Cd group. RNA-seq analysis revealed that 410 genes displayed differential expression (≥1.5-fold) in the 60 mg/kg supplementation group, compared to the control group. GO and KEGG pathway analysis results showed that Cd affected many genes involved in mitochondria and ion transport. In conclusion, this study elaborates the mechanisms underlying renal toxicity caused by Cd, which might provide target candidate genes for alleviating Cd poisoning in laying hens.

Keywords Cadmium · Laying hens · Renal damage · Antioxidant · Gene expression · Nephrotoxicity

Introduction
Cadmium (Cd) is a toxic heavy metal pollutant that poses great health risks to human, animals, and plants. Cd is ranked the seventh in the priority list of hazardous substances of the Agency for Toxic Substances and Disease Registry (ATSDR, 2019). Cd is ubiquitous in the environment which is derived from natural occurrence, industrial, and agricultural sources. Volcanic activity, erosion and abrasion of rocks and soil, forest fire, and mining, smelting (Casado et al. 2008), as well as from corrosive reagent, stabilisers in polyvinyl chloride (PVC) products, colour pigments and Ni–Cd batteries (Genchi et al. 2020), along with sewage sludge disposal and the application of phosphate fertiliser (Meng et al. 2018), results in the deposition of Cd in the environment, especially in soil. Cd is easily absorbed by plants from soil, while root crops, cereals, and grains especially readily take up Cd from soil. The European Food Safety Authority (EFSA) (2012) reported that grains and grain products, vegetables and vegetable products, and starchy roots and tubers ranked the highest contribution to total Cd intake in Europe. It can be inferred that the feedstuff contributes greatly to the Cd content in formula feed, since plant feedstuffs are the main components in formula feed. However, contribution of Cd derived from mineral feed cannot be ignored, since Cd naturally exists...
in sulphide ores of zinc, lead, and copper (Genchi et al. 2020). Constant ingestion of Cd causes serious health risks to human and domestic animals. Cd enters the blood via erythrocytes and albumin and then accumulates primarily in the kidney and liver with a long half-life (Satarug 2018). Hepatic metallothionein (MT) efficiently binds Cd in the liver as Cd-MT complexes, which protect the liver from Cd. After the complexes are distributed to the kidney, Cd is subjected to glomerular filtration, and then the complexes are dissociated into Cd, which is excreted in the urine (Genchi et al. 2020). Once the Cd content exceeds the binding capacity of MT, unconjugated Cd accumulates in the proximal tubules of the kidney and thereby leads to renal damage (Liu et al. 2015). Furthermore, Cd exposure may increase the production of reactive oxygen species (ROS) and therefore induces damage and death in kidney cells (Shi et al. 2017; Abdeen et al. 2019). Over generation of ROS and free radicals can lead to oxidative stress, disturb the antioxidant system, and induce apoptosis (Stohs et al. 2000; Lee et al. 2006; Ye et al. 2007). Following accumulation of ROS in proximal tubular cells, Cd may further induce apoptosis by the loss of mitochondrial membrane potential (Wang et al. 2009). SH-containing proteins can combine with Cd and form Cd-SH complex. However, the Cd-SH complex may impair mitochondrial function and result in oxidative damage (Abdeen et al. 2017). The proximal tubule of the kidney is the main site of calcium reabsorption, so exposure with Cd may lead to disturbances in Ca balance in the body. The nutritional requirements of Ca and other mineral elements is relatively high in laying hens to assure bone and eggshell quality, and consequently more mineral feed is supplemented in the formula feed of laying hens, which increases the risk of Cd exposure to laying hens. However, little information about the renal toxicity of Cd and the underlying molecular mechanism is available. Considering the cell-death and apoptosis triggered by Cd exposure, it is necessary to observe the ultrastructure and identify the abnormal organelles of damaged cells. In this work, transmission electron microscopy technology was used to reveal ultrastructural change after renal exposure. Transcriptome analysis plays an important role in identifying genetic networks, deciphering genome structure and function, establishing molecular biomarkers that respond to diseases and pathogens (Jiang et al. 2015). In the present study, RNA-sequencing bioinformatics analysis was applied to identify the key genes and pathway of Cd action in kidney of laying hens.

Domestic animals are easily exposed to Cd, especially via feed. Since the kidney is the primary cumulative (Bernhoft 2013) and target (Luo et al 2017) organ, as a result, kidney damage results in disturbance of ion reabsorption or transportation. While the nutritional requirements of Ca and other mineral elements of laying hens in peak egg production period is relatively high, the nephrotoxicity of Cd might affect the eggshell. However, information about potential risk of Cd on laying hens is limited. The purpose of this study was to clarify the effect of dietary Cd on eggshell quality and renal damage of laying hens in the peak laying period, by means of transcriptome technology and transmission electron microscope, the present study will provide a better understanding of the mechanism of renal toxicity in laying hens caused by Cd.

**Materials and methods**

**Animals treatment and experimental design**

The animal experimental was carried out under the guidelines of the Scientific Ethics Committee of Huazhong Agricultural University (Ethical Approval Code HZAUCH-2017–010). A total of 72 Hy-line brown laying hens at 40 weeks of age were randomly assigned to four treatments for 6 weeks, while each treatment included six replicates of three hens each, and the laying hens of each replicate were of the same age, and with insignificant difference of the average body weight (2.0 ±0.4 kg/bird). Laying hens were reared in conventional three-layer cages with three chickens in each cage. During the experiment, the laying hens received 16 h of illumination, and the temperature in the house was kept at 15–25 °C. After 7 days of acclimation, each group was fed with the basal diet incorporated with 0, 15, 30, and 60 mg Cd/kg for 6 weeks, and Cd was added as Cd chloride (CdCl₂·2.5H₂O, Sinopharm Chemical Reagent Co., Ltd.). The basal diet was formulated to meet the nutritional requirements of laying hens (NRC 1994), and the formula and its nutritional contents of the basal diet was published in our previous study (Tao et al. 2020), and the concentration of calcium and cadmium in basal diet was 3.9% and 1.21 μg/kg, respectively. After 6 weeks of Cd exposure, one hen from each replicate (six hens per treatment) was euthanised with carbon dioxide, and then blood was collected from the carotid artery, followed by quick removal of the kidneys. All the kidney tissue was rinsed with ice-cold deionised water, most of which was divided into aliquots, snap-frozen in liquid nitrogen, and preserved at -80 °C until analysis, and the rest of which were fixed in 1% osmium tetroxide in 0.1 M veronal buffer, pH 7.2, at 4 °C for transmission electron microscopic examination and fixed in formalin for haematoxylin and eosin (H&E) for microscopic slides (Luo et al. 2019a, b).

**Measurement of eggshell quality**

After 6 weeks of Cd exposure, six eggs of each replicate were collected. Eggshell strength and eggshell thickness were determined according to previous study (Mu et al. 2019). Eggshell strength was determined using an eggshell

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force gauge (EFG-0503, Robotmation Co., Ltd., Tokyo, Japan). The eggshell thickness was determined using a Vernier calliper at three points, the blunt end, equator, and sharp end, and the eggshell thickness was expressed as the average thickness of the three points.

**Concentration of Cd, Fe, Zn, Cu and Mn in the kidney**

The residue of Cd in kidney was measured by graphite furnace atomic absorption spectrometry (iCE 3500 GFAA, Thermo Fisher, USA), after wet digestion with HCl and HNO₃ (Sinopharm Chemical Reagent Co., Ltd, 7647–01-0, 7697–37-2). According to the method with minor revision as described by AOAC (AOAC, 2005 Proc. No 968.08) and Qu’s study (Qu et al. 2020), Cd was determined at λ = 228.8 nm, and test slip was 0.2 nm.

Kidney tissue samples were determined for Fe, Zn, Cu, and Mn by using flame atomic absorption spectrometry. The measurement wavelengths of iron, zinc, copper, and manganese were 248.3, 213.9, 324.8, and 279.5 nm, respectively. And the slit widths of Fe, Zn, Cu, and Mn were 0.2, 0.2, 0.5, and 0.2 nm, respectively. The standard solutions of Cd, Cu, and Fe were obtained from the National Institute of Metrology, China, while the standard solutions of Zn and Mn were obtained from the Institute for Environmental Reference Materials of Ministry of Environmental Protection, China.

**Renal histopathological and ultrastructure examination**

The renal histopathological examination was conducted as previously described by Zhang et al. (2016a, b). Fragments of kidney (1.0 × 0.5 × 0.5 cm blocks) were fixed and processed by routine paraffin embedding for histological evaluation. Sections of 4 μm in thickness were taken and stained with H&E for microscopic slides. The renal ultrastructural examination was carried out using transmission electron microscopy (Tecnai G2 20 TWIN, USA), and the adopted method was as previously described (Li et al. 2013). Kidney tissues (0.5 × 0.5 × 0.5 mm blocks) were excised immediately after euthanasia, and the tissue was prefixed in 2.5% glutaraldehyde solution and then rinsed with PBS (0.1 M) and then postfixed in 1% osmic acid at 4 °C for 30 min; next, the tissue was rinsed with PBS (0.1 M) three times. Then the tissue was subjected to dehydration with series of gradient concentration of alcohol and embedding in epoxy resin. Ultrathin sections were post-stained with uranyl acetate and lead citrate. Specimens were then examined under the transmission electron microscope.

**Serum biochemical indices analysis**

According to the method adopted by Zhang and Chen (Zhang et al. 2018; Chen et al. 2018), serum was prepared by centrifugation of the whole blood at 3000 r/min for 10 min at 4 °C and preserved at −80 °C until analysis. Serum concentrations of aspartate albumin (ALB), serum total proteins (TP), creatinine (CRE), and blood urea nitrogen (BUN) were analysed using an automatic biochemistry analyser (7100, Hitachi, Japan).

**Renal antioxidant status analysis**

Renal antioxidant status parameters, including the activities of glutathione peroxide (GPX), catalase (CAT) and total antioxidant capacity (T-AOC) and concentration of reduced glutathione (GSH) and malondialdehyde (MDA), were determined according to our previous study (Tao et al. 2020). The renal tissue samples (0.5 g) were thawed in 4.5 mL isotonic saline and homogenised on ice, and the supernatants were centrifuged at 12,000 g for 15 min at 4 °C. The antioxidant parameters of kidney were detected using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer’s instructions. Protein concentrations were determined by the bicinchoninic acid assay.

**Gene ontology enrichment analysis and KEGG enrichment analysis**

According to Luo (2019), gene function was described by using three databases including Clusters of Orthologous
Groups of Proteins, Kyoto Encyclopedia of Genes and Genomes (KEGG) Ortholog database, and Gene Ontology (GO). Differential expression analysis of gene (DEG) was conducted using the DESeq method, in such a transcript was considered to have significant DE if the \( P \) value < 0.05.

**Quantitative PCR**

As previously described (Zhang et al. 2016a, b), the mRNA levels of CACAN1D, CDC14A, GADD45B, G6PC2, GHR, EPHA2, IPAR3, WNT5B, CREB3L1, and FGF22 were determined using real-time qPCR (CFX384, Bio-Rad, CA, USA). The primers of the above genes and housekeeping gene \( \beta\)-actin are shown in Table 2. Relative abundance was analysed by the \( 2^{-\Delta\Delta C_t} \) method. Data were normalised with housekeeping gene \( \beta\)-actin.

**Statistical analysis**

Data were analysed by one-way ANOVA using SPSS 19.0 (SPSS, Inc., Chicago, IL, USA). First, the normal distribution was confirmed. Then Duncan’s multiple comparisons test was used to test the significant differences between treatment average values. The test results are presented as mean ± standard division (SD). \( P < 0.05 \) was considered statistically significant.

**Results**

**Dietary Cd exposure changed eggshell traits**

Eggshell quality is an important factor related to egg hatching, storage, and transport, and the results of eggshell strength and thickness after 6 weeks of 15, 30, and 60 mg/kg Cd exposure are shown in Fig. 1. As shown in Fig. 1, eggshell strength was not influenced by 6 weeks of Cd exposure, while 60 mg/kg Cd exposure significantly reduced eggshell thickness (\( P < 0.05 \)).

**Dietary Cd exposure changed the concentration of Cd, Fe, Zn, Cu, and Mn in kidneys of laying hens**

The results of residual Cd in kidneys of laying hens showed that dietary exposure of Cd at 15 mg/kg, 30 mg/kg, and 60 mg/kg significantly increased the residue of Cd in kidney (\( P < 0.01 \)) in a dose-dependent manner (Fig. 2A), with a significant positive association between dietary Cd concentration and residual Cd in the kidneys of laying hens (\( y = 1.9638x + 4.584, R^2 = 0.9812 \)).

Cd exposure also disturbed the renal ion balance. The concentration of Zn was significantly increased at the dose of 15, 30, and 60 mg/kg Cd (\( P < 0.01 \)). Meanwhile, the concentration of Fe was significantly reduced with supplementation at 30 and 60 mg/kg Cd (\( P < 0.05 \)). However, there was no significant difference in the contents of Cu and Mn in the kidney among the different treatment groups (Fig. 2B).

**Dietary Cd exposure changed serum biochemistry, renal histopathological, and ultrastructure**

At week 6, the serum concentrations of TP in the 15 mg/kg and 30 mg/kg Cd treatment groups were significantly decreased (\( P < 0.05 \)), while in the 60 mg/kg treatment group, the TP level was not significantly different from that of the control group. Dietary Cd exposure did not affect the concentration of ALB, BUN, and CREA (Table 1).

Compared with the control group, the kidney tissue showed consistent histopathological changes in the 15, 30, and 60 mg/kg Cd treatment groups, including lymphocyte infiltration in the stroma, proliferation of mesangial cells and vascular endothelial cells, and diffuse vesicular degeneration of renal tubular epithelial cells (Fig. 3). Based on the
RESULTS OF TRANSMISSION ELECTRON MICROSCOPY, ULTRASTRUCTURAL VARIATIONS IN RENAL CELLS WERE OBSERVED. COMPARED WITH THE CONTROL GROUP, THE MITOCHONDRIA SWELL ED SLIGHTLY, AND THE CRISTAE EXPANDED MILDLY AT 15 mg/kg Cd TREATMENT. AND THE MITOCHONDRIA SWELLED MODERATELY IN 30 mg/kg Cd TREATMENT. IN 60 mg/kg Cd TREATMENT, CHROMATIN CONDENSATION AND MAR-GINATION WERE FOUND. AT THE SAME TIME, CRISTAE EXPANSION AND MITOCHONDRIAL SWELLING WERE AGGRAVATED, COMPANIED WITH INCREASED ELECTRON DENSITY (Fig. 4). THE RESULTS SHOWED THAT Cd EXPOSURE IN THE KIDNEY DAMAGED THE MITOCHONDRIA OF RENAL CELLS AND CONSEQUENTLY IMPAIRED THE GLOMERULI AND

Table 1 Effects of Cd in diet on serum biochemical indexes of laying hens

| Indexes | Cadmium Concentration (mg/kg) | 0    | 15   | 30   | 60  |
|---------|-------------------------------|------|------|------|-----|
| ALB (g/L) | 19.4 ± 0.6 | 19.3 ± 0.9 | 19.4 ± 0.9 | 20.4 ± 0.6 |
| TP (g/L)  | 47.1 ± 2.3  | 40.1 ± 3.2  | 41.7 ± 3.9  | 47.8 ± 2.2  |
| BUN (mmol/L) | 0.20 ± 0.06 | 0.18 ± 0.08 | 0.26 ± 0.08 | 0.20 ± 0.03 |
| CREA (mmol/L) | 30 ± 3.0  | 29 ± 1.7    | 31 ± 0.0    | 32 ± 1.0    |

Notes: Values are expressed as means ± SD (n = 6), labelled means in a row without a common letter differ, P < 0.05

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| ALB (g/L) | 19.4 ± 0.6 | 19.3 ± 0.9 | 19.4 ± 0.9 | 20.4 ± 0.6 |
| TP (g/L)  | 47.1 ± 2.3  | 40.1 ± 3.2  | 41.7 ± 3.9  | 47.8 ± 2.2  |
| BUN (mmol/L) | 0.20 ± 0.06 | 0.18 ± 0.08 | 0.26 ± 0.08 | 0.20 ± 0.03 |
| CREA (mmol/L) | 30 ± 3.0  | 29 ± 1.7    | 31 ± 0.0    | 32 ± 1.0    |

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renal tubules. In addition, higher dose of Cd exposure caused
nuclear pyknosis, which probably lead to apoptosis.

**Dietary Cd exposure reduced the renal antioxidant
capacity of laying hens**

The effects of Cd exposure on renal activities of GPX, CAT,
and T-AOC and the content of GSH and MDA are shown in Table 2. Compared with the control group, the activity of GPX was significantly reduced by 25%, 35%, and 38%, respectively ($P < 0.05$) at the doses of 15, 30, and 60 mg/kg Cd treatment, while the activity of CAT was significantly reduced 29%, 20%, and 30% ($P < 0.05$), respectively. In addition, the activity of T-AOC was decreased by 88% ($P < 0.05$) with 60 mg/kg Cd exposure. However, there was no significant difference in the concentration of GSH and MDA among these four groups (Table 2). The detection of antioxidant indexes of kidney showed that Cd in the diet affected the antioxidant enzymes of the kidney and the redox system balance of the kidney in laying hens.

**Sequencing, de novo assembly, and annotation**

Considering the production performance (Tao et al. 2020)
and the damage of kidney caused by Cd, the control group
and 60 mg/kg Cd treatment group were chosen to be under RNA-seq analysis. A statistical summary of RNA-seq on the kidney following 60 mg/kg Cd treatment is shown in Table 3. A total of 60,664,221 and 59,865,041 raw reads with Q20 values ranging from 97 to 98% were collected from the control and Cd treatment groups, respectively. After filtering adapters and trimming ambiguous and low-quality reads, there were 60,121,211 and 59,337,449 high-quality and clean reads obtained, accounting for 99% and

![Fig.4](image) Effects of different dietary Cd supplementation on kidney ultrastructure of laying hens in week 6. a Renal ultrastructure in 0 mg/kg Cd group. b Renal ultrastructure in 15 mg/kg Cd group. The mitochondria swelled slightly and the cristae expanded mildly. c Renal ultrastructure in 30 mg/kg Cd group. The mitochondria swelled moderately. d Renal ultrastructure in 60 mg/kg Cd group. Chromatin condensation and margination were found. Cristae expansion and mitochondrial swelling were aggravated, accompanied with increased electron density (N, nucleus; green arrows, mitochondria; bar, 1 μm; magnified 5000×)

| Indexes                  | Cadmium Concentration (mg/kg) |
|-------------------------|--------------------------------|
|                         | 0                | 15               | 30               | 60               |
| GSH (mg/g prot)         | 2.13 ± 0.33      | 1.80 ± 0.43      | 1.86 ± 0.27      | 1.86 ± 0.73      |
| GPX (U/mg prot)         | 54.24 ± 2.74$^a$ | 40.50 ± 3.26$^b$ | 35.30 ± 6.05$^b$ | 33.52 ± 4.40$^b$ |
| CAT (U/mg prot)         | 45.66 ± 4.56$^a$ | 32.58 ± 2.92$^b$ | 36.69 ± 1.84$^b$ | 31.74 ± 3.83$^b$ |
| T-AOC (U/mg prot)       | 0.41 ± 0.13$^a$  | 0.32 ± 0.03$^a$  | 0.32 ± 0.06$^c$  | 0.05 ± 0.01$^b$  |
| MDA (nmol/mg prot)      | 0.28 ± 0.01      | 0.26 ± 0.03      | 0.27 ± 0.03      | 0.31 ± 0.04      |

Notes: Values are expressed as means ± SD ($n = 6$), labelled means in a row without a common letter differ, $P < 0.05$
99% of the total raw reads, respectively. Consequently, high-quality clean reads were further mapped onto the *Gallus gallus* genome (Ensembl Database) using the TopHat2 tool. The average ratio of high-quality reads in comparison with the reference genome was 94% (Table 3), and 24,881 genes were aligned to the database (Supplemental file 2).

**Dietary Cd exposure changed renal transcriptome expression**

Differential expression (DE) analysis indicated that dietary exposure of 60 mg/kg Cd induced responsive genes in the kidneys of laying hens. Compared with the control group, there were 410 transcripts showing 1.5-fold or greater ($P < 0.05$) DEGs in 60 mg/kg Cd exposure group. Among them, 221 genes were up-regulated, and 189 genes were down-regulated in the Cd exposure treatment. The full list of DEGs is listed in Supplemental file 2.

### Table 3 Statistical summary of RNA-seq on kidney with 60 mg/kg Cd exposure for 6 weeks

| Sample | RAW Reads number | Bases(Gb) | Q20 value\(^{a}\) | Corrected Reads number | Reads ratio | Total mapped reads percentage | Uniquely mapped reads percentage |
|--------|------------------|-----------|------------------|------------------------|------------|-------------------------------|---------------------------------|
| Control-1 | 55,725,380       | 8.4       | 97.41            | 55,170,218             | 99.00%     | 93.49%                        | 88.91%                          |
| Control-2 | 61,349,430       | 9.3       | 97.68            | 60,834,222             | 99.16%     | 93.86%                        | 89.18%                          |
| Control-3 | 64,917,854       | 9.8       | 97.18            | 64,359,192             | 99.14%     | 93.97%                        | 89.39%                          |
| 60 mg/kg Cd-1 | 58,911,598       | 8.9       | 97.46            | 58,381,778             | 99.10%     | 93.48%                        | 88.98%                          |
| 60 mg/kg Cd-2 | 52,963,106       | 8.0       | 97.61            | 52,483,368             | 99.09%     | 92.98%                        | 88.42%                          |
| 60 mg/kg Cd-3 | 67,720,420       | 1.0       | 97.55            | 67,147,202             | 99.15%     | 93.78%                        | 89.24%                          |

\(^{a}\)Q20 value means the sequencing quality values that correspond to 1% chance of error

Fig. 5 GO annotation analysis of differential genes in kidney of laying hen. The top 10 GO terms based on biological process ranked by fold-enrichment. More changes appeared with the genes related to single organism process, cellular process, metabolic process, and biological regulation. The top 10 GO terms based on cellular component were related to cell part, membrane and organelle. The top 5 GO terms based on molecular function, genes with binding function and catalytic activity have more changes.

The top 10 groups of biological process, top 10 groups of cellular component, and top 5 groups of molecular function are shown in Fig. 5. In the biological process category, the most abundant groups covered single organism process, cellular process, metabolic process, biological process, and regulation of biological process. As for the cellular component category, the most abundant groups were cell, cell part, membrane, organelle, and membrane part. In the molecular function, binding, catalytic activity, transporter activity, molecular transducer activity, and signal transduction activity were the most highly expressed GO terms. The DEGs related to the GO and KEGG pathway results involved in mitochondria and ion transport are shown in Table 4.

The real-time qPCR (Fig. 6) results show that renal mRNA levels of *EPHA2, LPAR3, WNT5B, CREB3L1*, and *FGF22* were significantly increased ($P < 0.05$) in 60 mg/kg Cd treatment, while the mRNA expression levels of *CDC14A, CACNA1D, GADD45B, G6PC2*, and *GHR* in

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the dietary Cd treatment (≥ 15 mg/kg) were significantly decreased (P < 0.05), compared to that of the control group, which were in agreement with the RNA-seq results.

**Discussion**

The eggshell thickness was decreased with 60 mg/kg Cd dietary exposure treatment, which suggests that dietary Cd exposure could disrupt calcium metabolism. It was previously reported that, accompanying renal tubular injury, Cd exposure reduced the concentration of plasma 1,25(OH)2D3 and the absorption of calcium by inhibiting the synthesis of calcium binding protein (Ferraro et al., 2011; Li et al. 2017). Furthermore, since the renal tubule is an important site of calcium absorption, the damage to the renal tubule caused by Cd may be an important reason for the decrease in calcium deposition in eggshells, which resulted in a decrease in eggshell thickness. The transcriptomic results also indicate that genes related to calcium ion transmembrane transport, such as *CACNA1D*, were decreased. Cav1.3 is encoded by *CACNA1D* and belongs to the family of voltage-gated L-type Ca2+ channels; *CACNA1D* missense mutations permit enhanced Ca2+ signalling through Cav1.3 (Pinggera and Striessnig 2016).

The renal Cd residue in laying hens was positively correlated with the concentration of dietary Cd, which was in accordance with a previous study that 31-week-old laying hens ingesting dietary Cd at 150 mg/kg for 90 days resulted in a residual Cd level in kidneys 400 times higher than that of the control group (Zhang et al. 2018). Dietary Cd exposure also disturbed the renal ion balance of laying hens. The reason may be that Cd can compete with some essential nutrients for the same transmembrane carrier and therefore disturb the ion balance (Shi et al. 2017). The RNA-seq results showed that there were many genes related to ion transport that had changed in the 60 mg/kg Cd group. Zn is an essential trace element and is the component of more than 300 enzymes related to cell metabolism and gene expression. Cd can replace Zn and bind to MT, resulting in the redistribution of Zn in the cytoplasm and serum (Xia et al. 2016). Previous studies have reported that 1-day-old broiler chickens exposed to 100 mg/kg Cd had higher concentrations of Cu and lower concentrations of Mn and Fe in the kidney (Al-Waeli et al. 2012). Our results show that Cd can accumulate in the kidney and disturb the balance of Zn and Fe in the kidney. The difference in renal ion disturbance induced by Cd exposure may be associated with differences in animal ages and Cd dosages.

Kidney lesions were found in laying hens after exposure to dietary Cd at concentrations of 15, 30, and 60 mg/kg. Laying hens manifested typical symptoms of renal injury, including lymphocyte infiltration in the stroma, proliferation of mesangial cells and vascular endothelial cells, and diffuse vesicular degeneration of renal tubular epithelial cells, along with swelling of mitochondria and expansion

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**Table 4** The differentially expressed genes grouped by GO and KEGG enrichment analysis

| Gene ID          | Gene name | FC    | P value |
|------------------|-----------|-------|---------|
| Mitochondria     |           |       |         |
| ENSGALG00000006361 | NEU4      | 1.834 | **      |
| ENSGALG00000007945 | CRYAB     | 1.803 | ***     |
| ENSGALG00000008121 | CYP17A1   | 1.703 | **      |
| ENSGALG00000004777 | AIFM2     | 1.62  | **      |
| ENSGALG00000027188 | SREBF1    | 1.578 | **      |
| ENSGALG00000009442 | ETFDH     | 0.635 | ***     |
| ENSGALG00000015053 | GLDC      | 0.626 | **      |
| ENSGALG00000002960 | HMGCS2    | 0.616 | **      |
| ENSGALG00000009700 | PDK4      | 0.533 | ***     |
| ENSGALG00000028967 | DNAJC27   | 0.468 | ***     |
| Ion transport    |           |       |         |
| ENSGALG00000026089 | SLC26A6   | 1.796 | **      |
| ENSGALG00000006638 | GRIK4     | 1.737 | **      |
| ENSGALG00000001661 | SLCA22A    | 1.678 | **      |
| ENSGALG00000005631 | TRPM2     | 1.646 | **      |
| ENSGALG000000017168 | SLN       | 1.562 | *       |
| ENSGALG00000005995 | SLC13A5   | 0.661 | *       |
| ENSGALG00000005332 | CACNA1D   | 0.557 | **      |

*P < 0.05; **P < 0.01, and ***P < 0.001

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![Fig.6](image-url) Effects of dietary Cd on the mRNA expression levels of *CACNA1D*, *CDC14A*, *GADD45B*, *G6PC2*, *EPHA2*, *LARP3*, *WNT5B*, *CREB3L1*, *FGF22*, and *GHR* in the kidney of laying hens in week 6. Values are means (n = 6/group), with standard deviation represented by vertical bars. Different letter means *P < 0.05
of cristae. Renal damage was aggravated with an increase in the dietary Cd concentration. Our results are consistent with previous research showing that Cd exposure caused glomerular fibrosis, tubular stenosis, and mitochondrial and endoplasmic reticulum swelling (Shi et al. 2017; Wang et al. 2018). The mitochondrial respiratory chain is crucial to maintain energy homeostasis through oxidative phosphorylation, generating adenosine triphosphate (ATP), which is the energy necessary for life. Additionally, mitochondria also function in the synthesis of amino acids, lipids and phospholipids, ion homeostasis, motility, and in apoptosis (Genchi et al. 2020). Cd may damage mitochondria, manifested by altered Ca$^{2+}$ signalling (Biagioli et al. 2008) and increased mitochondrial ROS formation (Belyaeva et al. 2006). Mitochondrial morphological changes mediated by Cd involve post-translational modifications, such as phosphorylation, ubiquitination, and sumoylation (Soubannier and McBride 2009). These results show that Cd exposure at concentration above 15 mg/kg for 6 weeks could induce renal histopathological changes in laying hens, manifested as lymphocytic infiltration and vesicular degeneration.

Cd inhibits ATPase, lactate dehydrogenase (LDH), superoxide dismutase (SOD), and glutathione peroxidase (GPx) activities and additionally enhances ROS activity and lipid peroxidation (Cannino et al. 2009); similar results were observed in the current study. Although Cd cations are unable to generate free radicals directly, Cd exposure promotes the production of ROS, including superoxide radicals, hydrogen peroxide, and hydroxyl radicals (Genchi et al. 2020). Excessive ROS production leads to macromolecule oxidation with free radical attack on phospholipids, thus altering the integrity of mitochondrial membranes, mitochondrial membrane depolarisation, and leading to mtDNA mutation (Ott et al. 2007). ROS are normally eliminated by the enzymatic (SOD, CAT, GPx) and non-enzymatic (GSH, vitamin C, vitamin E) antioxidants. Cd also reacts with exogenous and endogenous antioxidants (Cuypers et al. 2010), which result in the accumulation of ROS and aggravation of the redox imbalance.

The RNA-seq results also revealed that mitochondrial damage contributed greatly to renal damage caused by Cd. The GO and KEGG pathway analysis results showed that Cd affected many genes, some of which are involved in mitochondria and ion transport. The top upregulated gene was NEU4. NEU4 is targeted to mitochondria and can regulate several cancer-associated glycans (Cai et al. 2019). The most downregulated gene was DNAJC27, belonging to the HSP40 family and with modulatory activity on the HSP70 family, which is crucial to protein synthesis in mitochondria (Jores et al. 2018). Furthermore, many genes were involved in apoptosis and inflammation, which were subjected to further assessment. The mRNA expression levels of these genes, such as WNT5B (related to the Wnt signalling pathway and a significant regulator of cancer), was significantly upregulated in the 60 mg/kg Cd supplemented group. Moreover, TNF binding to TNFR1 triggers receptor trimerisation and leads to the assembly of the TNFR1-associated signalling complex (complex I), which passes through a series of reactions that activate NF-κB and MAPK signalling (Webster and Vucic, 2020). The MAPK pathway plays an important role in phosphorylation and hormone regulation (Lee et al. 2016) and was significantly increased by Cd treatment. In addition, the mRNA expression level of genes related to cellular growth and apoptosis was significantly lower than that of the control group. Our study indicates that the marked fluctuations in mRNA expression of inflammation-related and growth/apoptosis-related genes may be the result of renal damage induced by Cd exposure.

**Conclusion**

In summary, the present experiments in laying Cd-exposed hens demonstrated that dietary Cd exposure reduced the eggshell thickness and increased the residual Cd in the kidney. Cd induced structural and ultrastructural abnormalities in the kidney, which was characterised by damage to glomeruli and renal tubules, and the deformation of nuclei and mitochondria, respectively. Dietary exposure to Cd also induced oxidative stress, manifested by reduced activities of renal antioxidant enzymes. From the RNA-seq results, it can be inferred that the mechanism underlying the renal toxicity caused by Cd is that Cd affects the expression of genes involved in mitochondria and ion transport. Our studies provide an association between the Cd-induced renal toxicity in laying hens and adverse effects on eggshells, but further studies are required to reveal the precise signalling mechanism by which Cd affects mitochondrial function in the kidneys of laying hens.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s11356-021-18405-2.

**Acknowledgements** I would like to thank Pei Zhang and Du An-na from The Core Facility and Technical Support, Wuhan Institute of Virology, for their help with producing TEM micrographs.

**Author contribution** Man Zhao and Wenbo He: Data curation. Writing—original draft. Can Tao and Beiyu Zhang: Methodology, Data curation. Shuai Wang: Supervision. Zhangjian Sun: Conceptualisation, Project administration, Writing—review and editing.

**Funding** This study was supported by the National Key Research and Development Program of China (Project No. 2016YFD0501208).

**Availability of data and materials** All data generated or analysed during this study are included in this published article and its supplementary information files.
Declarations

Ethics approval The animal experimental was carried out under the guidelines of the Scientific Ethics Committee of Huazhong Agricultural University (Ethical Approval Code HZAUCH-2017-010).

Consent to participate Not applicable.

Conflict of interest The authors declare no competing interests.

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