Protective effect of nano-selenium on mercury-induced prehierarchical follicular atresia in laying hens

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ABSTRACT This study investigated the effect of nano-selenium (**nano-Se**) in protecting laying hens from mercury (**Hg**)-induced prehierarchical follicular atresia. Furthermore, the endoplasmic reticulum stress (**ERS**) was explored to reveal the molecular mechanism. In vivo, 720 Hyline-Brown laying hens were treated with Hg and nano-Se alone or in combination. In vitro, the prehierarchical follicles were treated with Hg, nano-Se and 4-phenyl butyric acid (**4-PBA**) alone or in combination (Control, 25 μM Hg group, 10 μM nano-Se group, 20 μM nano-Se group, 25 μM Hg + 10 μM nano-Se group, 25 μM Hg + 20 μM nano-Se group, 25 μM Hg + 4-PBA group, and 25 μM Hg + 20 μM nano-Se + 4-PBA group). The GCs were treated with Hg and nano-Se alone or in combination (Control, 15 μM Hg group, 6 μM nano-Se group, 12 μM nano-Se group, 15 μM Hg + 6 μM nano-Se group, 15 μM Hg + 12 μM nano-Se group). The results revealed that dietary Hg significantly reduced laying performance ($P < 0.05$) and egg quality ($P < 0.05$), whereas nano-Se addition prevented these reductions ($P < 0.05$). Hg exposure significantly induced the accumulation of Hg in PHFs ($P < 0.05$), prehierarchical follicular atresia ($P < 0.05$) and apoptosis in PHFs, whereas nano-Se addition significantly prevented these effects ($P < 0.05$). The levels of sex hormones ($P < 0.05$) were significantly decreased after Hg exposure in vivo and in vitro, while nano-Se addition prevented the reductions. Furthermore, the RNA-Seq results showed that the key factors of the ERS presented differential expression, including C/EBP homologous protein, protein kinase RNA-like endoplasmic reticulum kinase (***PERK***) and activating transcription factor 6 (***ATF6***) in GCs. Hg exposure significantly increased the key gene expression of endoplasmic reticulum stress in GCs, whereas nano-Se addition prevented the induction of expression of these genes. In addition, the protein levels of PERK, inositol requiring protein 1α (**IRE1α**) and ATF6 were significantly increased, whereas nano-Se addition prevented the enhancements of protein expression in GCs. In conclusion, this study shows that Hg exposure can reduce induce prehierarchical follicular atresia, whereas nano-Se can prevent these effects. Our results also elucidate a key role of ERS in these protective effects of nano-Se in laying hens.

Key words: follicular atresia, laying hens, mercury, nano-selenium

INTRODUCTION

Mercury (**Hg**), as a highly toxic heavy metal, can accumulate chronically in tissues (Azevedo et al., 2012). Currently, exposure to Hg in poultry feedstuff is still at a high risk. The Feed Health Standard (GB13078-2017) stipulates that the Hg content in chicken compound feedstuff shall not exceed 100.00 μg/kg in China. However, due to the uneven levels of raw material batches, the content of Hg in some of the complete feedstuffs (especially in stone powder and calcium hydrogen phosphate) is much higher than the health standard of feed in China (Yin et al., 2017). Previous studies found that dietary Hg exposure could lead to accumulation of Hg in the ovary and significantly reduce laying performance in laying hens (Ma et al., 2018a, 2018c). Prehierarchical follicular atresia plays a fundamental role in laying performance and the fundamental cause of follicular atresia is apoptosis in prehierarchical follicles (**PHFs**) (Yao et al., 2020). Therefore, Hg-induced apoptosis in PHFs will be the key to revealing the molecular mechanism of Hg interference on performance in laying hens.

Hg can induce endoplasmic reticulum stress (**ERS**) to induce apoptosis in mammals (Carranza-Rosales et al., 2005). Activating transcription factor 6 (**ATF6**), protein kinase RNA-like endoplasmic reticulum kinase (**PERK**), and inositol requiring protein 1 pathways are key pathways of ERS in apoptosis (Logu et al., 2013). In

the process of apoptosis, B-cell lymphoma-2 associate X (**Bax**) is the key molecule of pro-apoptotic, while Bcl-2 is the key molecule of anti-apoptotic (Green and Reed, 1998; Gross et al., 1999; Riedl and Shi, 2004). However, the association of Hg exposure and induction apoptosis of follicular granulosa cells (**GCs**) in laying hens and the role of ERS is previously unknown.

Selenium (**Se**) is a trace element with antioxidant capacity in animals (Rotruck et al., 1973). As we know, the Se content is 0.1 to 0.25 mg/kg in the diet of laying hens. Nano-selenium (**Nano-Se**) is a selenium source feed additive that eliminates the excessive ROS in cells (Zhang et al., 2008). Se plays an important role in the protection of normal ovarian development (Bozkurt et al., 2012). To date, the protective mechanism of nano-selenium on Hg-induced apoptosis in PHFs in laying hens has not been reported, and it is still unclear whether ERS is involved in this process.

This study investigated the effect of Hg on prehierarchical follicular atresia and the protective effect of nano-selenium in laying hens. Furthermore, the correlation of ERS with apoptosis in PHFs was explored to discover the molecular mechanism by which nano-Se protects against Hg-induced prehierarchical follicular atresia.

MATERIALS AND METHODS

The experiments were conducted following the standards for administration of experimental practices and the regulations for the administration of affairs concerning experimental animals. All experiment were conducted following the animal use guidelines established by the Henan University of Science and Technology, China (approve number: 063-2022).

Investigation of Hg Content in Poultry Feedstuff in China

This investigation study of Hg content was performed in 14 provinces of China, including Hebei, Shanxi, Henan, Gansu, Heilongjiang, Jinlin, Qinghai, Liaoning, Sichuan, Guizhou, Hubei, Hunan, Yunnan, and Guangxi Province. A total of 280 poultry farms (20 farms were surveyed in each province) were randomly selected and investigated from the 14 provinces of China. The survey data contained the Hg content in complete formulated feed. The Hg content in the feedstuff was determined by cold-vapor atomic absorption spectrometry (Garrido et al., 2004). The excess rate of Hg content in feedstuff was calculated according to a previous study (Ma et al., 2022).

Animals and Treatments With Chemicals in Vivo

In an in vivo experiment, a total of 720 Hyline-Brown laying hens with similar laying performances at 45 wk of age were raised in a chicken farm in Luoyang, China. The birds were allocated into 6 treatments, and each group had 6 replicates with 20 birds per replicate. The 6 experimental groups were as follows: Control group; 27.00 mg/kg Hg group (**27Hg**); 0.25 mg/kg nano-Se group (**0.25Se**); 0.50 mg/kg nano-Se group (**0.5Se**); 27.00 mg/kg Hg + 0.25 mg/kg nano-Se group (**27Hg +0.25Se**); and 27.00 mg/kg Hg + 0.50 mg/kg nano-Se group (**27Hg+0.5Se**). Both Hg and nano-Se were obtained from Tongjie Chemical reagent Co. Ltd. (Yuncheng, China). Hg was added in the form of mercuric chloride with 99.7% purity, and nano-Se had 99.5% purity. Both the composition of the basal diet and nutrient content are presented in Supplement Table 1. The Hg levels in the experimental diets was determined by cold-vapor atomic absorption spectrometry and shown in Appendix 1 (Garrido et al., 2004). The birds were kept in cages (1 bird per cage with $0.30\ \mathrm{m}^3$) with free access to water and feed for 10 wk with relative humidity between 62 and 70%, temperature between 24 and 27°C and illumination at 16 h/day (20 lx). In an in vitro experiment, a total of 32 Hyline-White laying hens at 45 wk of age were obtained from a chicken farm in Luoyang, China. Feeding management was the same as in the in vivo experiments.

Laying Performance and Egg Quality

Laying performance included egg weight (**EW**), henday egg production (**EP**), and feed intake, which were recorded daily. Both egg weight and feed intake were determined using a scale (ME2002TE/02, Meilen Instrument Co., Ltd, Shanghai, China). A total of 36 eggs in each group (6 eggs of each replicate) were gathered for the measurements of eggshell strength, yolk color, albumen height, and Haugh unit, which were measured by an egg-quality metric. The eggshell thickness was measured by a micrometer caliper (EA-01, ORKA, Israel).

Primary Culture of PHFs

Ovaries were removed from the chickens and the PHFs were washed with ice-cold sterile phosphate buffered saline (**PBS**). Primary culture of PHFs was performed according to a previous methodology (Ma et al., 2022).

Primary Culture of GCs

The GCs in PHFs were isolated and cultured based on a previous study (Ma et al., 2018c). In brief, the PHFs were isolated from the ovary and placed in a sterile cell culture dish containing PBS. The granulosa layer was separated from the PHFs and cut into approximately 1 mm^3 squares, and they were incubated with 0.2% collagenase (Biochannel, Nanjing, China) in a water bath at 37°C for 5 min. After the GCs were washed with sterile PBS, they were incubated in high glucose DMEM (Biochannel) containing 15% fetal bovine serum

Table 1. Investigation of Hg content in poultry feedstuff in China[1].

Province	Range(μg/kg)[2]	Average Hg content (μg/kg)	Over-limit ratio (%)[3]
Shaixi ND $\sim$ 34.27	28.35 $\pm$ 0.07	0.00	
Gansu ND $\sim$ 87.21	55.37 $\pm$ 0.08	0.00	
Qinghai	22.58 $\sim$ 108.29	106.98 $\pm$ 0.09	17.50
Sichuan	ND $\sim$ 39.54	23.57 $\pm$ 0.03	0.00
Guizhou	79.54 $\sim$ 203.75	103.45 $\pm$ 0.11	22.50
Henan	ND $\sim$ 55.64	19.36 $\pm$ 0.20	0.00
Hebei	ND $\sim$ 68.47	26.78 $\pm$ 0.14	0.00
Heilongjiang	32.54 $\sim$ 101.54	88.57$\pm$0.17	0.00
Jilin	11.54 $\sim$ 140.39	104.58 $\pm$ 0.11	10.00
Liaoning	ND $\sim$ 83.24	39.87 $\pm$ 0.68	0.00
Hubei	ND $\sim$ 29.75	14.35 $\pm$ 0.02	0.00
Hunan	ND $\sim$ 30.43	16.54 $\pm$ 0.02	0.00
Yunnan	89.32 $\sim$ 108.44	101.32 $\pm$ 0.51	16.67
Guangxi	80.04 $\sim$ 113.66	107.53 $\pm$ 0.77	16.67

[1]Hg = mercury; the Hg content in poultry feedstuff should not exceed 100 μg/kg in Feed Hygiene Standard (GB13078-2017) of China;
[2]ND = not detected.
[3]According to the national standard for the standard rate (Over-limit ratio, % = the number of exceeded samples/the total number of samples $\times$ 100%), n = 40.

(Biochannel) in a 5% CO_2 and humidified 95% air incubator at 37°C.

Treatments with Chemicals in Vitro

For detection of the protective effect of nano-Se on Hg-induced prehierarchical follicular atresia, the PHFs were treated with Hg, nano-Se and 4-phenyl butyric acid (**4-PBA**, an endoplasmic reticulum stress inhibitor) alone or in combination [Control, 25 μM Hg group (**25Hg**), 10 μM nano-Se group (**10nano-Se**), 20 μM nano-Se group (**20nano-Se**), 25 μM Hg + 10 μM nano-Se group (**25Hg+10nano-Se**), 25 μM Hg + 20 μM nano-Se group (**25Hg+20nano-Se**), 25 μM Hg + 4-PBA group (**25Hg+4-PBA**), and 25 μM Hg + 20 μM nano-Se + 4-PBA group (**25Hg+20nano-Se+4-PBA**)]. 4-PBA was obtained from Sigma Chemical Co. Ltd. (Louis, MO).

For detection of the protective effect of nano-Se on Hg-induced apoptosis in GCs, the GCs were treated with Hg and nano-Se and alone or in combination [Control, 15 μM Hg group (**15Hg**), 6 μM nano-Se group (**6nano-Se**), 12 μM nano-Se group (**12nano-Se**), 15 μM Hg + 6 μM nano-Se group (**15Hg+6nano-Se**), 15 μM Hg + 12 μM nano-Se group (**15Hg+12nano-Se**)].

Determination of Sex Hormone Profiles

After the rearing experiment, the birds were euthanized. The blood samples were collected and transferred to the laboratory on ice within hours. The serum samples were isolated by centrifugation at 2,500 $\times$ g for 15 min at 4°C for the measurement of sex hormone profiles. PHFs were treated with their allocated group treatment for 24 h. Progesterone (**P4**), luteinizing hormone (**LH**), estradiol (**E$_2$**) and follicle-stimulating hormone (**FSH**) are important indicators for the evaluation of ovarian function. The sex hormone profiles in serum and PHFs were determined by radioimmunoassay kits (LanpaiBio, Shaihai, China).

Determination of Hg in the PHF

The accumulation of Hg in the PHFs was measured by cold atomic absorption spectrometry according to a

**Table 2.** Effects of dietary nano-Se addition on laying performance and egg quality in Hg-containing diets.

Items[1]	Control	0.25Se	0.5Se	27Hg	27Hg+0.25Se	27Hg+0.5Se
Laying performance						
EP, %	88.92 $\pm$ 1.94[a]	87.93 $\pm$ 1.77[a]	87.54 $\pm$ 1.66[a]	74.31 $\pm$ 2.27[b]	75.43 $\pm$ 2.02[b]	85.54 $\pm$ 1.66[a]
EW, g	61.11 $\pm$ 1.49[a]	61.37 $\pm$ 1.88[a]	62.37 $\pm$ 1.94[a]	55.09 $\pm$ 1.09[b]	60.37 $\pm$ 1.88[a]	62.11 $\pm$ 1.84[a]
Feed intake	115.43 $\pm$ 1.62	112.42 $\pm$ 1.48	117.22 $\pm$ 1.38	114.11 $\pm$ 1.60	118.31 $\pm$ 1.09	116.33 $\pm$ 1.87
FCR	1.89 $\pm$ 0.14[b]	1.83 $\pm$ 0.11[b]	1.88 $\pm$ 0.10[b]	2.07 $\pm$ 0.19[a]	1.96 $\pm$ 0.12[a]	1.87 $\pm$ 0.14[b]
Egg quality						
Haugh units	84.93 $\pm$ 1.35[a]	86.39 $\pm$ 1.48[a]	86.49 $\pm$ 2.01[a]	77.85$\pm$ 1.54[b]	76.18 $\pm$ 1.87[b]	82.48 $\pm$ 1.38[a]
Albumen height	8.18 $\pm$ 0.14[a]	8.13 $\pm$ 0.15[a]	8.23 $\pm$ 0.14[a]	6.83 $\pm$ 0.23[b]	7.17 $\pm$ 0.17[b]	8.27 $\pm$ 0.22[a]
Yolk color	7.44 $\pm$ 0.22	7.34 $\pm$ 0.51	7.33 $\pm$ 0.55	7.27 $\pm$ 0.26	7.42 $\pm$ 0.40	7.19 $\pm$ 0.28
Eggshell strength	3.69 $\pm$ 0.16	3.77 $\pm$ 0.22	3.72 $\pm$ 0.22	3.74 $\pm$ 0.21	3.73 $\pm$ 0.19	3.78 $\pm$ 0.28
Eggshell thickness	0.38 $\pm$ 0.01[a]	0.38 $\pm$ 0.01[a]	0.38 $\pm$ 0.01[a]	0.31 $\pm$ 0.01[b]	0.32 $\pm$ 0.01[b]	0.38 $\pm$ 0.01[a]

[1]Abbreviations: EP, hen-day egg production; EW, egg weight; FCR, feed conversion ratio; kgf/m^2, kilogram-force/m^2.
[a,b]Means with different superscript letters differ significantly in the same row ($P < 0.05$). Values are the means $\pm$ SE (n = 8).
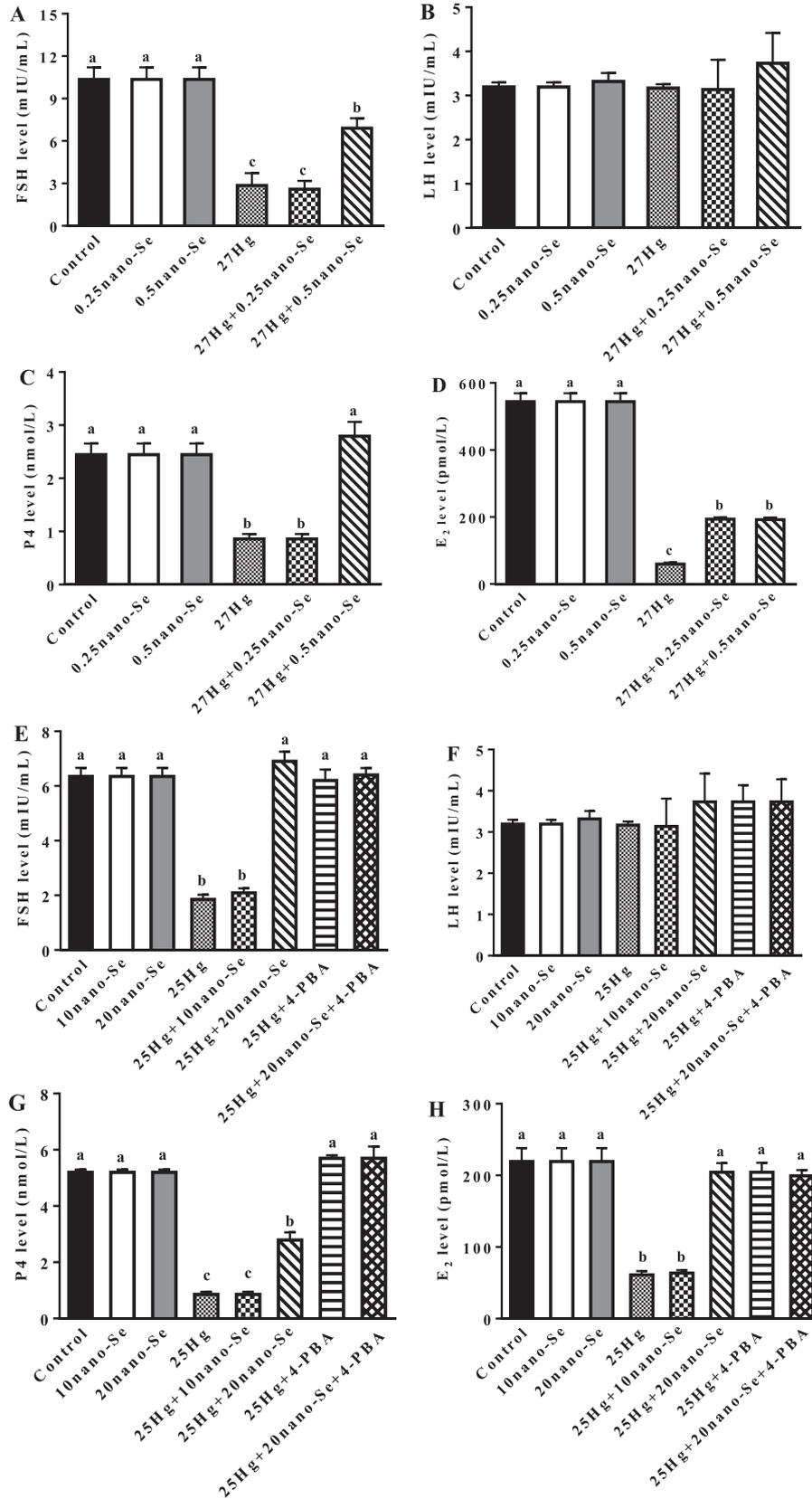
Figure 1. Effects of nano-Se addition on sex hormone levels affected by Hg exposure in vitro and vivo. (A) FSH in vivo; (B) LH level in vivo; (C) P4 level in vivo; (D) E_2 level in vivo; (E) FSH in vitro; (F) LH level in vitro; (G) P4 level in vitro; (H) E_2 level in vitro. Abbreviations: 4-PBA, 4-phenyl butyric acid; FSH, follicle-stimulating hormone; LH, luteinizing hormone; P4, progesterone; E_2, estradiol. Values are the means $\pm$ SE (n = 4). [a-c]Means with different superscripts differ significantly among any groups ($P < 0.05$).
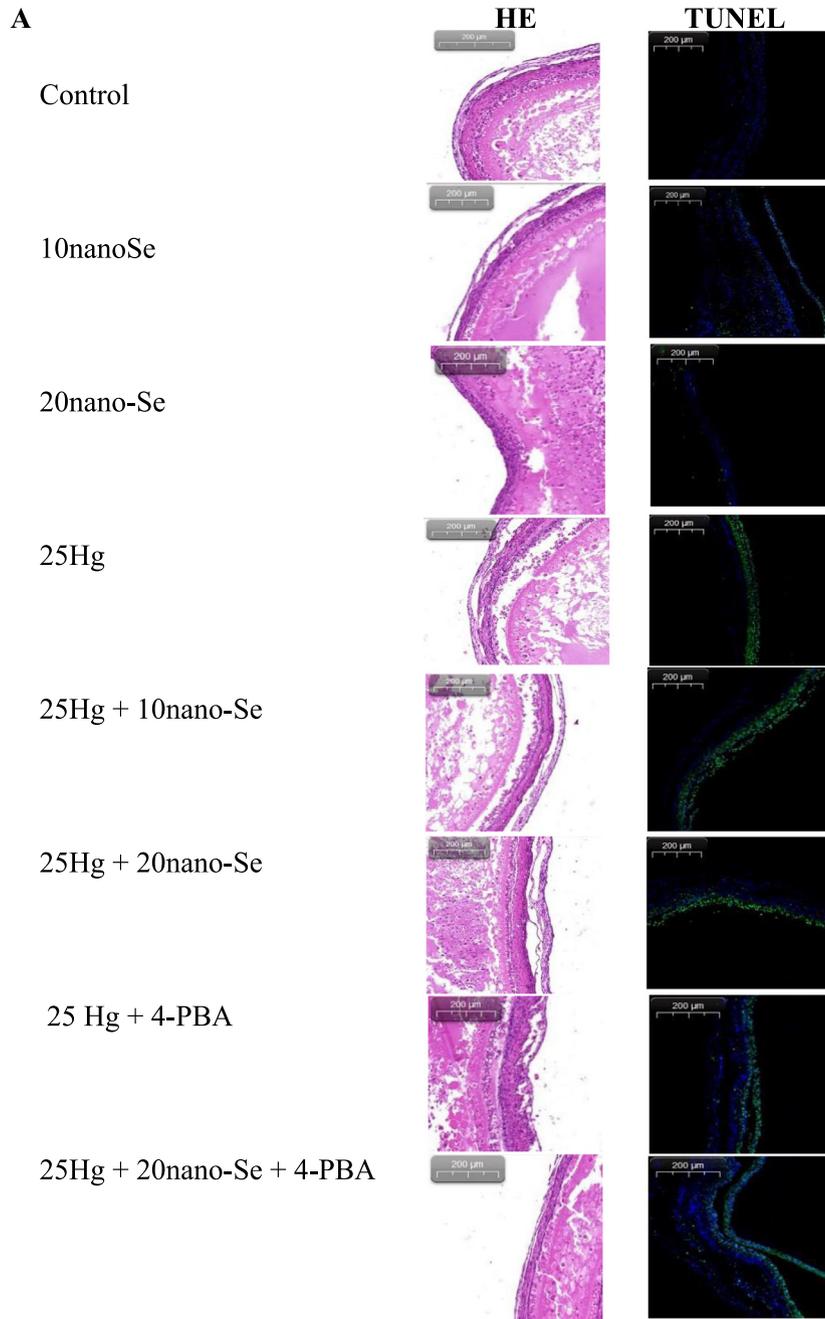
Figure 2. Effect of nano-Se addition on Hg-induced follicular atresia and cell apoptosis in PHFs of laying hens. (A) H&E staining and TUNEL assay in the PHFs (Scale bar: 200 μm for H&E staining and 200 μm for TUNEL assay); (B) accumulation of Hg in PHFs; (C) prehierarchical follicular atresia rate; (D) fold increase of apoptosis in PHFs of laying hens. Abbreviations: 4-PBA, 4-phenyl butyric acid; nano-Se, nano-selenium; Hg, mercury; PHFs, prehierarchical follicles. Values are means $\pm$ SE (n = 4). [a-c]Means with different superscripts differ significantly among any groups ($P < 0.05$).

previous study (Garrido et al., 2004). The results are presented as micrograms per gram dry weight.

Follicular Morphological Observation Assay

The PHFs were fixed in 4% paraformaldehyde and dehydrated with graded ethanol followed by trimming and embedding in paraffin at 55°C. The method of paraffin section preparation for follicular samples and

determination of follicular atresia were based on a previous reference (Ma et al., 2018b).

TUNEL Assay

The PHFs were treated with their allocated group treatment for 24 h. In parallel, the PHFs were exposed for 24 h in the presence or absence of 1 μM 4-PBA. TUNEL staining of PHFs was conducted according to instructions of a TUNEL kit. Cell apoptosis in PHFs
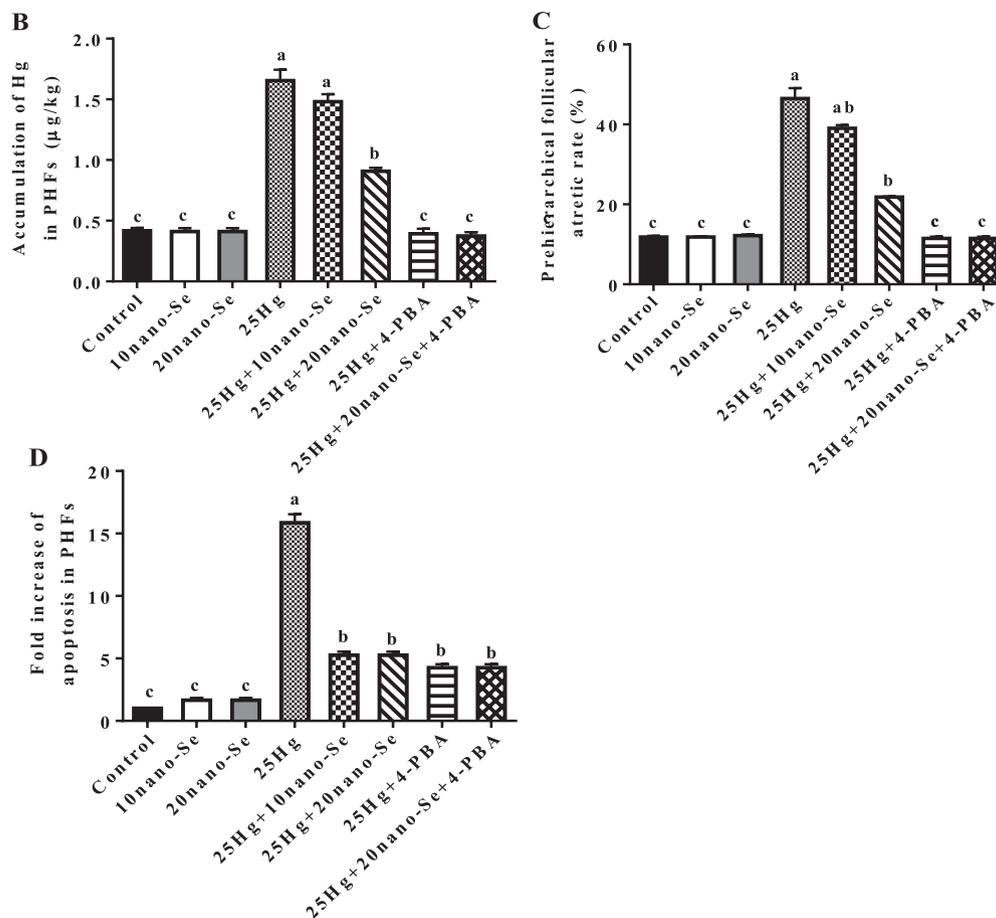
Figure 2 Continued.

was observed and quantitatively analyzed at 40 × magnifications under a fluorescence microscope (Leica, Wetzlar, Germany).

RNA Extraction, Real-Time Quantitative PCR, and RNA Seq Analysis

The GCs were treated with their allocated group treatment for 24 h. In parallel, the GCs were exposed for 24 h in the presence or absence of 1 μM 4-PBA. An RNAiso Kit (Biomaker, Beijing, China) was used to extract the total RNA of PHFs, which was used to generate cDNA using a cDNA synthesis kit (Biomaker). qRT-PCR was performed using a SYBR Premix Ex Taq kit (Biomaker) on a CFX96 Touch PCR detection system (Hercules, CA). PCR amplication was performed under the following conditions: 95°C for 26 s followed by 41 cycles of 93.5°C for 5 s, 59°C for 20 s, and 71.5°C for 30 s. Comparisons of expression levels were measured by the $2^{-\Delta\Delta Ct}$ method normalized to β-actin (Livak and Schmittgen, 2002). The forward and reverse primers of *PERK, activating transcription factor* (***ATF4***), *ATF6, C/EBP homologous protein* (***CHOP***), *apoptosis signal-regulating*

kinase 1 (***ASK1***), *tumor necrosis factor-associated factor 2* (***TRAF2***), *inositol requiring protein 1α* (***IRE1α***), and *housekeeping gene* (***GAPDH***) are presented in Supplement Data 1. RNA-seq in GCs was performed based on a previous study (Ma et al., 2020).

Western Blot Analysis

Proteins were extracted from the GCs and quantified by a commercially kits (Servicebio technology, Wuhan, China). The antibodies of PERK (1:500, GB11510), IRE1α (1:500, GB11685), and ATF6 (1:500, GB11297) were purchased from Servicebio technology Co. Ltd. The western blot analysis was performed based on a previous study (Ma et al., 2020b).

Statistical Analysis

All data are shown as the mean ± standard error (**SE**) and were analyzed by one-way ANOVA. When means in different groups were significantly different ($P < 0.05$), analysis of Tukey's multiple comparisons was conducted with SPSS version 21.0 (SPSS Inc., Chicago, IL).
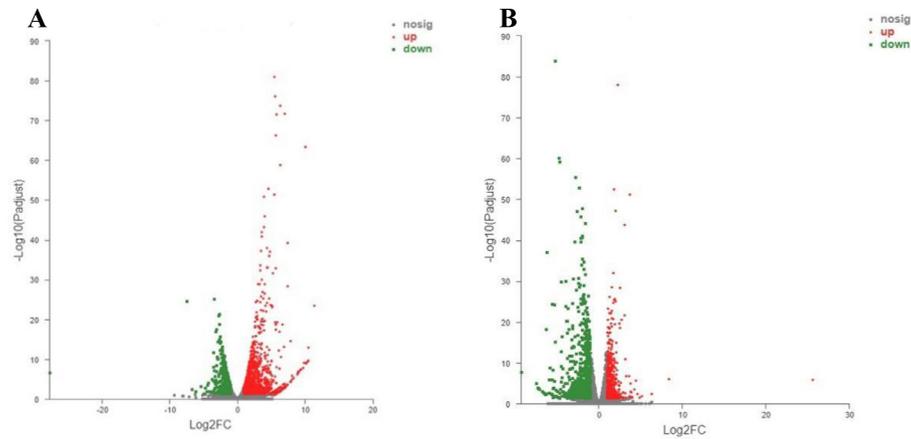
C. Control vs 15Hg

Gene ID	Gene name	Log₂FC	*P* adj
Upregulated genes			
Gene-CHOP	CHOP	11.41	3.67E-24
Gene-PERK	PERK	10.57	1.20E-13
Gene-PAPD7	PAPD7	10.47	2.66E-10
Gene-ATF6	ATF6	10.40	3.73E-10
Gene-TRPM1	TRPM1	10.22	1.25-09
Downregulated genes			
Gene-PATZ1	PATZ1	-27.74	0.033652
Gene-TESC	TESC	-6.14	6.44-E10
Gene-CPNE9	CPNE9	-4.69	0.023099
Gene-TCN2	TCN2	-4.49	4.95E-05
Gene-ZCCHC4	ZCCHC4	-3.68	0.001045

Figure 3. The RNA-seq analysis of effects of nano-Se addition on ERS pathway affected by Hg exposure in GCs. (A) Volcano of differentially expressed genes (Control vs. 15Hg); (B) volcano of differentially expressed genes (15Hg vs. 15Hg+12nano-Se); (C) differentially expressed genes in GCs (Control vs. 15Hg); differentially expressed genes in GCs (15Hg vs. 15Hg+12nano-Se).

RESULTS

Investigation of Hg Contents in Poultry Feedstuff in Different Provinces of China

In 14 provinces of China, the average Hg contents in the complete formula feedstuff of laying hens in Qinghai, Guizhou, Jilin, Yunnan, and Guangxi were beyond the 100 μg/kg, and the over-limit ratios were 17.50%, 22.50%, 10.00%, 16.67%, and 16.67%, respectively. In addition, the average Hg contents in the complete formulated feed of laying hens in Shanxi, Gansu, Sichuan, Henan, Hebei, Heilongjiang, Liaoning, Hubei and Hunan did not exceed the 100 μg/kg, and the laying rates were 87.55 to 92.13% (Table 1).

D. 15Hg vs 15Hg+12nano-Se

Gene ID	Gene name	Log$_2$FC	P adj
Upregulated genes			
Gene-MYL3	MYL3	25.60	0.004143
Gene-RNF216	RNF216	6.29	1.24E-13
Gene-TRPM1	TRPM1	5.17	0.010973
Gene- DNAJB1	DNAJB1	4.87	4.05E-56
Gene- FRMPD3	FRMPD3	4.60	0.005184
Downregulated genes			
Gene-CHOP	CHOP	-9.24	2.74E-08
Gene-ZNF385B	ZNF385B	-7.45	1.20E-05
Gene-MT3	MT3	-7.30	0.000161
Gene-PERK	PERK	-7.22	0.000254
Gene-ATF6	ATF6	-7.09	0.000372

Figure 3 Continued.

Dietary Nano-Se Addition Prevented the Reductions of Egg Quality and Laying Performance Induced by Hg Exposure

Dietary 27 mg/kg Hg addition significantly reduced EP and EW compared with the other groups ($P < 0.05$), while dietary 0.50 mg/kg nano-Se addition completely prevented these reductions ($P < 0.05$). However, dietary 27 mg/kg Hg addition significantly enhanced the FCR compared with the other groups ($P < 0.05$), while dietary 0.50 mg/kg nano-Se addition prevented the increase of FCR ($P < 0.05$; Table 2).

Dietary 27 mg/kg Hg addition significantly decreased Haugh units ($P < 0.05$), albumen height ($P < 0.05$) and eggshell thickness ($P < 0.05$) compared with other groups, while dietary 0.50 mg/kg nano-Se addition completely prevented these reductions ($P < 0.05$; Table 2).

Nano-Se Alleviated the Reductions of Sex Hormone Levels Induced by Hg Exposure

In vivo, dietary 27 mg/kg Hg addition significantly decreased the levels of FSH ($P < 0.05$), E$_2$ ($P < 0.05$), and P4 ($P < 0.05$), while dietary 0.50 mg/kg nano-Se addition alleviated the reductions of FSH ($P < 0.05$), E$_2$ ($P < 0.05$), and P4 ($P < 0.05$; Figures 1A and 1B). In

vitro, the levels of FSH ($P < 0.05$), E$_2$ ($P < 0.05$), and P4 ($P < 0.05$) were significantly decreased in the 25Hg group compared with the control group. However, 20 μM nano-Se addition prevented these reductions ($P < 0.05$; Figures 1C and 1D). In addition, Hg and nano-Se additions did not affect the LH level in vitro or in vivo.

### Nano-Se Alleviated the Hg-Induced Follicular Atresia and Cell Apoptosis in PHFs

The atresia rate and apoptotic cells in the nano-Se group were significantly lower as compared to the 25Hg group. Compared with the 25Hg group, the enhancement of follicular atresia rate and cell apoptosis in PHFs of laying hens were completely maintained in the 25Hg + 4-PBA group and the 25Hg + 20nano-Se + 4-PBA group (Figure 2).

Nano-Se Changed the Differentially Expressed Gene Profiles Affected by Hg in GCs

Based on the volcano of differentially expressed genes in GCs, the expression of many genes changed after Hg exposure and nano-Se addition. For example, after Hg exposure, the expression levels of CHOP, PERK, and ATF6 were upregulated, while the expression levels of
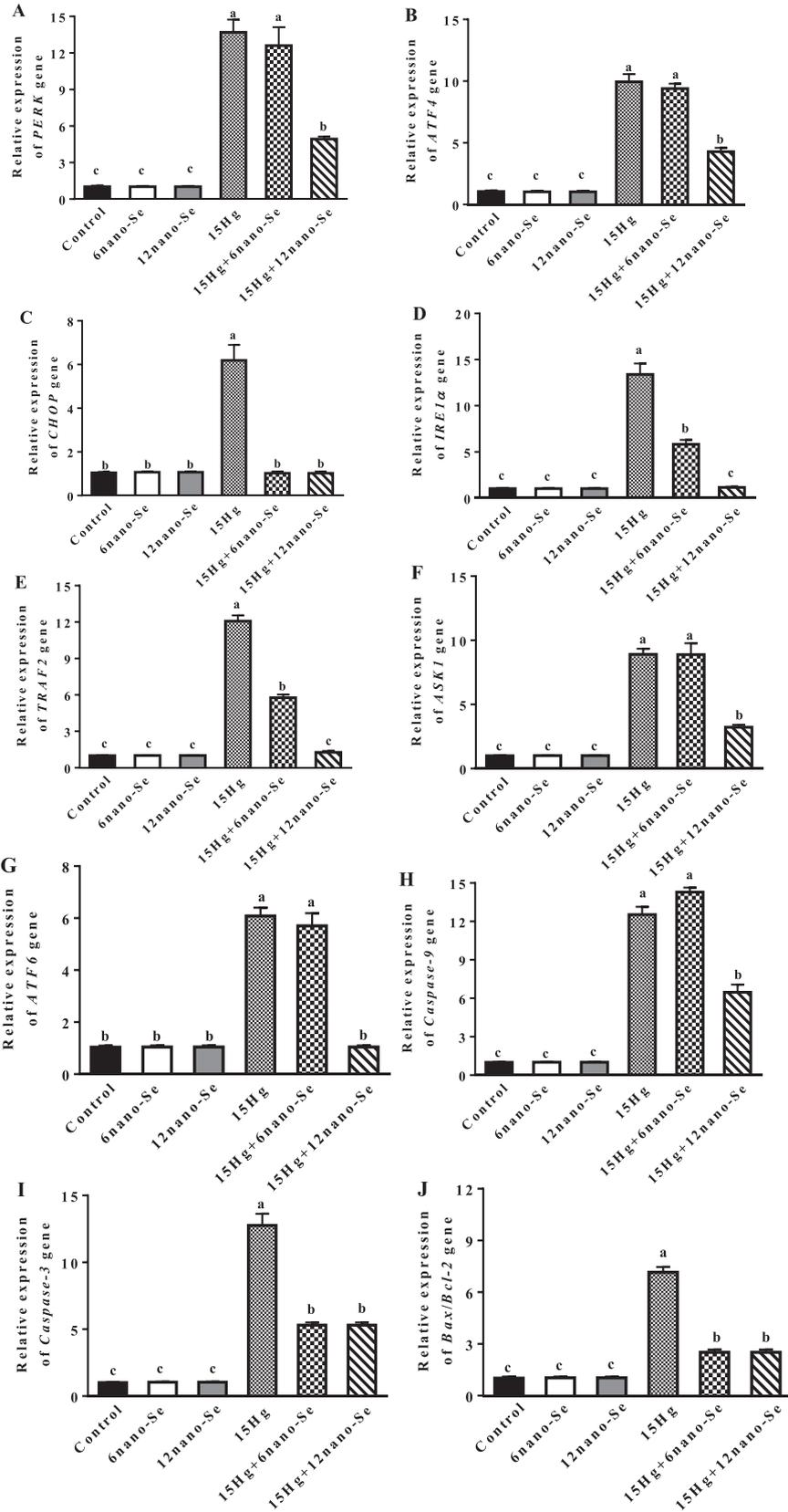
Figure 4. Effects of nano-Se addition on gene expressions of ERS pathway affected by Hg exposure in GCs. (A) Relative expression of *PERK* gene; (B) relative expression of *ATF4* gene; (C) relative expression of *CHOP* gene; (D) relative expression of *IRE1α* gene; (E) relative expression of *TRAF2* gene; (F) relative expression of *ASK1* gene; (G) relative expression of *ATF6* gene; (H) relative expression of *Caspase-9* gene; (I) relative expression of *Caspase-3* gene; (J) relative expression of *Bax/Bcl-2* gene. Abbreviations: ERS, endoplasmic reticulum stress; GCs, granulosa cells; *PERK, protein kinase RNA-like endoplasmic reticulum kinase; ATF4, activating transcription factor 4; CHOP, C/EBP homologous protein; IRE1α, inositol-requiring enzyme 1α; TRAF2, tumor necrosis factor-associated factor 2; ASK1, apoptosis signal-regulating kinase 1; ATF6, activating transcription factor 6; Bax, B-cell lymphoma-2 associate X.* Values are the means ± SE (n = 8). [a-c]Means with different superscripts differ significantly among any groups ($P < 0.05$).
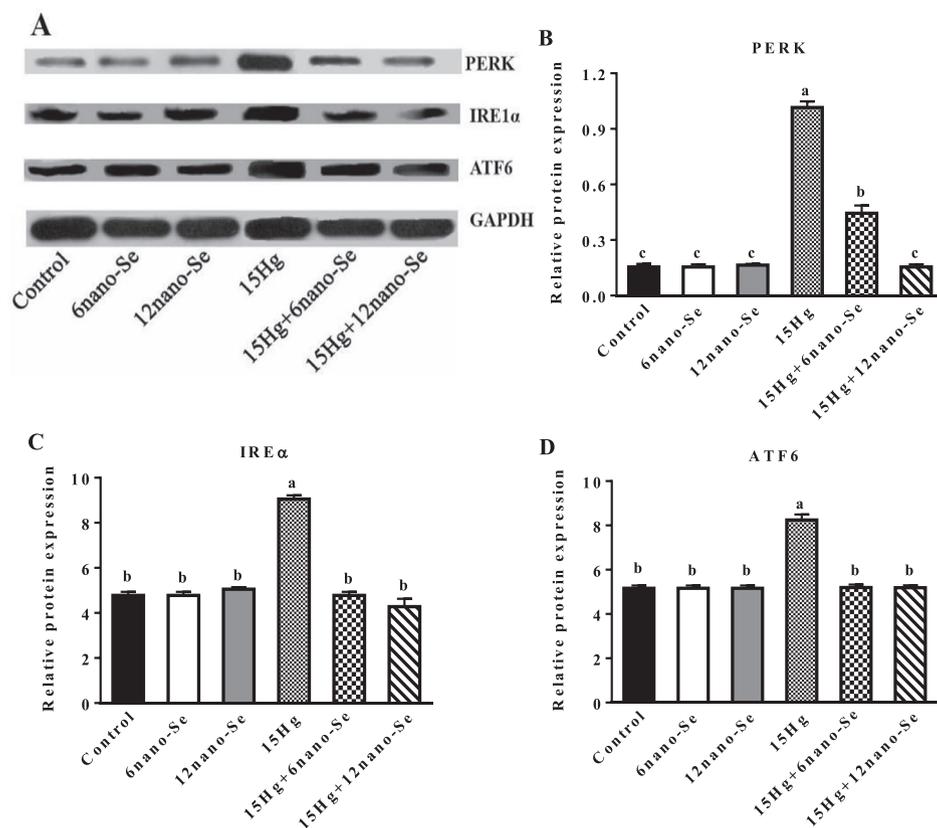
Figure 5. Effects of nano-Se addition on the key protein expressions of ERS pathway affected by Hg exposure in GCs. (A) The protein expressions of PERK, IRE1α and ATF6 were determined by Western blot; (B) relative protein expression of PERK; (C) relative protein expression of IRE1α; (D) relative protein expression of ATF6. Abbreviations: ERS, endoplasmic reticulum stress; GCs, granulosa cells; *PERK, protein kinase RNA-like endoplasmic reticulum kinase; IRE1α, inositol-requiring enzyme 1α; ATF6, activating transcription factor 6.* Values are the means ± SE (n = 8). [a–c]Means with different superscripts differ significantly among any groups ($P < 0.05$).

PATZ1, TESC, CPNE9, TCN2, and ZCCHC4 were downregulated. Compared with the 15Hg group, the expression levels of MYL3, RNF216, TRPM1, DNAJB1, and FRMPD3 were upregulated, while the expression levels of CHOP, PERK, and ATF6 were downregulated in the 15Hg + 12nano-Se group (Figure 3).

Nano-Se Prevented the Enhancements of Gene and Protein Expressions in the ER Stress Pathway in GCs

Compared with the control group, 15 μM Hg exposure significantly enhanced the expression of *IRE1α* ($P < 0.05$), *TRAF2* ($P < 0.05$), *ASK1* ($P < 0.05$), *PERK* ($P < 0.05$), *ATF4* ($P < 0.05$), *CHOP* ($P < 0.05$), *ATF6* ($P < 0.05$), *caspase-9* ($P < 0.05$), *caspase-3* ($P < 0.05$), and *Bax/Bcl-2* ($P < 0.05$), while 12 μM nano-Se prevented these expression patterns in GCs ($P < 0.05$). 6 μM nano-Se alleviated the Hg-induced increases in the expression levels of *CHOP* ($P < 0.05$), *IRE1α* ($P < 0.05$), *TRAF2* ($P < 0.05$), *caspase-3* ($P < 0.05$), and *Bax/Bcl-2* ($P < 0.05$) in GCs (Figure 4).

Compared with the control group, 15 μM Hg exposure significantly enhanced the protein expression of PERK ($P < 0.05$), IRE1α ($P < 0.05$) and ATF6 ($P < 0.05$), while both 6 μM and 12 μM nano-Se prevented this expression pattern in PHFs (Figure 5).

DISCUSSION

In recent years, exposure to Hg in poultry feedstuff has remained at a high risk (Yin et al., 2017). In this investigation, the average Hg contents in the complete formula feedstuff of laying hens in Qinghai, Guizhou, Jilin, Yunnan and Guangxi of China were beyond the 100 μg/kg, and the over-limit ratios were 17.50%, 22.50%, 10.00%, 16.67%, and 16.67%, respectively. These results indicated there is a great risk of exposure to Hg in the complete formula feedstuff of laying hens in China.

Previous studies showed that dietary Hg pollution could affect egg quality and laying performance in laying hens, which was in accordance with this study (Ma et al., 2018a, 2018c). In this study, dietary Hg exposure significantly decreased egg quality and laying performance, while significantly enhancing the enhancement of the follicular atresia rate in laying hens. Egg production can be significantly affected by follicular atresia, which has been proven in this study (Johnson, 2014). Nano-Se, a selenium source feed additive, has a protective effect on heavy metal poisoning in mammals (Zhang et al., 2020a). In this study, dietary nano-Se supplementation effectively alleviated the reduction of laying performance and egg quality. The protective effect of nano-selenium on laying rate could be achieved by preventing follicular atresia. Previous studies showed that dietary nano-Se
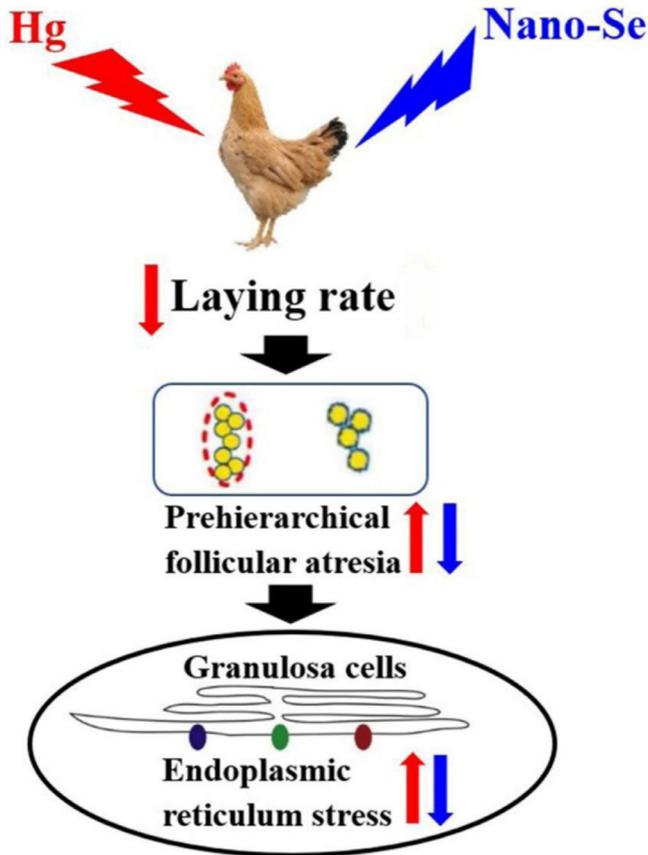
Figure 6. Endoplasmic reticulum stress in granulosa cells might play a key role in the reduction in laying rate induced by Hg and the protective effect of nano-Se in laying hens. Abbreviations: Hg, mercury; Nano-Se, nano-selenium.

supplementation could improve the broiler performance and prevent the reduction in performance and egg quality caused by Hg in laying hens, which was in accordance with this study (Cai et al., 2012; Ma et al., 2021). The laying rate of laying hens is significantly affected by prehierarchical follicular atresia and the fundamental cause of follicular atresia is apoptosis in GCs. In an in vitro study, Hg exposure in PHFs led to the enhancements of follicular atresia in PHFs and apoptosis in the granular layer of PHFs. These findings provide potential mechanism by which Hg interfered with the laying performance in this study. Similarly, the main reason for the protective effect of nano-Se on laying rate was that it alleviated apoptosis in GCs. In addition, follicular atresia is related to the secretion of sex hormones in laying hens. The secretion of sex hormones is closely correlated with the differentiation of follicular GCs and apoptosis of GCs (Shen et al., 2014). In this study, Hg exposure significantly reduced the secretion of sex hormones in vitro and in vivo, while nano-Se addition could prevent these decreases in sex hormones. The absence of the steroidal hormones in atretic follicles is due to the planned death of GCs, which renders them ineffective steroidal hormones. As a result, the atretic follicles loses their ability to make estrogen and becomes unresponsive to the LH and FSH hormones. Nevertheless, the nano-Se addition might alleviate the apoptosis in GCs by inhibition of ERS pathway, effectively alleviate follicular

atresia and eventually prevent the decreases in sex hormones. These also confirmed that Hg could induce prehierarchical follicular atresia and the protective effect of nano-Se in laying hens.

Many signaling pathways are closely related to cell apoptosis in GCs, including the ERS pathway. Once apoptosis is triggered in GCs, Bcl-2, and caspase are important conduction factors (Gross et al., 1999; Riedl and Shi, 2004). In this research, Hg exposure significantly increased the expression of *caspase-3, caspase-9*, and *Bax/Bcl-2*, while nano-Se addition prevented the increases in *caspase-9* and *Bax/Bcl-2*. This indicated that Hg exposure induced apoptosis in GCs and that nano-Se might alleviate this increase in apoptosis. We hypothesized that nano-Se might inhibit the occurrence of apoptosis in GCs by interfering with the key factors in caspase and Bcl-2 families, which could be proved by a previous study (Zhang et al., 2020b). To explore the molecular mechanism of the effect of Hg on apoptosis in GCs, RNA-seq analysis was performed. We found that the key factors of the ERS pathway showed differential expression, including CHOP, PERK, ATF6, and EIF4E2. When ERS is activated, the key factors active the CHOP transcription factor to trigger apoptosis in cells. In this study, we found that Hg exposure could significantly increase the key factors of ERS, including PERK, ATF4, ATF6, CHOP, ASK1, TRAF2, and IRE1α, while nano-Se addition could prevent these enhancements in PHFs and GCs. Previous studies reported that Hg exposure could induce apoptosis in GCs and renal cells by means of the ERS pathway in laying hens, which is in accordance with our results (Chen et al., 2017; Ma et al., 2020b; Ma et al., 2021). Other studies also supported the results of this study. They showed that heavy metal exposure induced apoptosis in GCs, liver and kidney and that there were protective effects of Se against heavy metal-induced toxicities via suppression the of ERS response in chickens (Zhang et al., 2008, 2020a; Liu et al., 2018; Zhu et al., 2021). As we know, when the ERS is activated, deposition of unfold proteins induces the separation of glucose regulated protein 78 from the three transmembrane receptors, including PERK, IRE1α, and ATF6 (Chen et al., 2019). We hypothesized that nano-Se addition recombined the glucose regulated protein 78 and the receptor factors and alleviated the ERS in GCs. In the view of the results, this study provides some new ideas for a central role of ERS in the reduction of laying rate induced by Hg and the protective effect of nano-Se in laying hens.

In conclusion, Hg exposure could reduce laying performance in laying hens. The main mechanism for the reduction in laying rate induced by Hg was its effects of increasing prehierarchical follicular atresia and apoptosis in PHFs. However, nano-Se protected laying hens from Hg-induced the reduction in laying rate by preventing follicular atresia and apoptosis in PHFs. Furthermore, ERS might play a key role in the reduction in laying rate induced by Hg and the protective effect of nano-Se in laying hens (Figure 6).

ACKNOWLEDGMENTS

DISCLOSURES

The authors declare that they have no conflicts of interest to this work entitled "Protective effect of nano-selenium on mercury-induced prehierarchical follicular atresia in laying hens". We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.102190.

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