Effects of varying dietary intoxication with lead on the performance and ovaries of laying hens

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ABSTRACT In this study, we explored the effect of dietary lead nitrate on zootechnical performance, egg quality, accumulation of ovarian plumbum (**Pb**), follicular atresia rate, and ovarian oxidative stress in laying hens. Furthermore, the nuclear factor erythroid 2–related factor 2 (**Nrf2**) signaling molecule was studied to reveal the molecular mechanism of the stress. A total of 512 Hy-Line Brown laying hens aged 40 wk were randomly allocated to 4 groups (with 8 pens per group and 16 hens per pen). The Pb concentrations used to treat the 4 groups were 3.20, 33.20, 63.20, and 93.20 mg/kg. The results revealed that dietary Pb exposure significantly linearly reduced the zootechnical performance ($P < 0.01$) but significantly linearly increased the feed conversion ratio ($P < 0.01$). The dietary Pb exposure significantly linearly reduced the Haugh units ($P < 0.01$), albumen height ($P < 0.01$), eggshell thickness ($P < 0.01$), and eggshell strength ($P < 0.01$). In addition, the dietary Pb exposure significantly enhanced the follicular atresia rate ($P < 0.01$). After dietary Pb exposure, superoxide dismutase ($P < 0.01$) and glutathione peroxidase (**GSH-Px**) ($P < 0.01$) activities and glutathione ($P < 0.01$) contents were significant decreased quadratically, and there were significant linear decreases in the activities of catalase (**CAT**) ($P < 0.01$) and glutathione reductase (**GR**) ($P < 0.01$), whereas malondialdehyde content was significantly linearly increased ($P < 0.01$). In addition, except for manganese superoxide dismutase, the gene expressions of copper–zinc superoxide dismutase ($P < 0.01$), CAT ($P < 0.01$), and GR ($P < 0.01$) were significant decreased linearly. In addition, there were significantly quadratic decreases in the mRNA expressions of GSH-Px ($P < 0.01$) and Nrf2 ($P < 0.01$). By way of contrast, the Kelch-like ECH-associated protein 1 (**Keap1**) gene expression was significantly linearly increased ($P < 0.01$). In conclusion, dietary Pb exposure could induce oxidative stress by impairing the Nrf2–Keap1 signal pathway in the ovaries of laying hens.

Key words: laying hen, lead, Nrf2–Keap1 pathway, ovary, oxidative stress

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INTRODUCTION

In recent years, environmental pollution has subjected humans and animals to various metal agents, including plumbum (**Pb**) (Jaishankar et al., 2014). Lead is a heavy metal acknowledged to have high toxicity, and it can be deposited in soils and oceans, which are the sources of Pb contamination in livestock diets (Caban and Rasmussen, 1994). Previous studies have reported that lead concentration in feeds around a metal production center showed a high probability of exceeding the livestock tolerance center (Farmer and Farmer, 2000). At present, there is still high risk of Pb exposure from livestock feed around the world.

Lead is known to be an unnecessary metal element in animals. Numerous health problems in humans and livestock can be caused by Pb pollution, including nephrosis, hepatopathy, neurological disorders, respiratory disorders, and reproductive disturbances (Lockitch, 1993; Papanikolaou et al., 2005). The mammalian reproductive system could be damaged by Pb exposure (Pinon-Lataillade et al., 1993; Nakade et al., 2015). In general, the symptoms of plumbism in the reproductive system were the thickening of the endometrium, the narrowing of the uterine lumen, and the alteration of testosterone secretion. In female animals, the ovaries are the key reproductive organs. Especially for laying hens, both zootechnical performance and egg quality have a close relationship to ovarian performance. A previous study showed that dietary mercury exposure reduced zootechnical performance and egg quality in laying hens (Ma et al., 2018a). However, there is still a lack of sufficient

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evidence to explain the toxicological effects of dietary Pb exposure on laying hens.

Previous studies have found that Pb exposure can produce reactive oxygen species and induce oxidative stress in animals. Under Pb exposure, the antioxidant enzymes in various vertebrate tissues would be depleted, including superoxide dismutase (**SOD**), glutathione peroxidase (**GSH-Px**), and catalase (**CAT**) (Ahamed and Siddiqui, 2007; Reglero et al., 2009). In addition, glutathione (**GSH**) plays a key role in antioxidant defense, and malondialdehyde (**MDA**) is a biomarker of oxidative stress (Patrick, 2006). When oxidative stress is triggered, the antioxidant enzyme activities are regulated by the upstream antioxidant enzyme genes in rats (Tiedge et al., 1997). The antioxidant enzyme genes are regulated by several intracellular transcription factors as well, such as nuclear factor erythroid 2–related factor 2 (**Nrf2**) (Kwak et al., 2001). In oxidative stress, Nrf2 separates from Kelch-like ECH-associated protein 1 (**Keap1**) in the cytoplasm, moves into the nucleus, and ultimately activates the mRNA expression of the downstream antioxidant enzyme (Zhang, 2006). Although the Nrf2–Keap1 signaling pathway has participated in biological defense mechanisms when exogenous Pb-induced oxidative stress affects rat livers, the functional mechanisms have not been clarified in the ovaries of laying hens (Long et al., 2016).

In this study, we investigated the effects of dietary lead nitrate exposure on zootechnical performance, egg quality, follicular atresia rate, and ovarian oxidative stress in laying hens. In addition, the expression of Nrf2–Keap1 molecules and downstream antioxidant genes in ovaries was further explored to reveal the underlying molecular mechanism.

MATERIALS AND METHODS

All the experiments performed on animals were implemented in accordance with Henan University of Science and Technology (Luoyang, China), with the approval of the Institutional Animal Care and Use Committee.

Birds, Diets, and Experimental Design

A total of five hundred twelve 40-wk-old Hy-Line Brown laying hens with similar performance, obtained from Hy-Line Poultry Breeding Technology Co. Ltd. in Beijing, China, were randomly allocated to 4 treatments. Each group was made up of 8 replicates of 16 hens. Four different graded dosages of Pb (in the form of lead nitrate) were added to a basal corn–soybean diet (Table 1) to create experimental diets containing 0, 30, 60, and 90 mg/kg of Pb, respectively. The birds were kept in cages (0.4 m^2 per chick), with 2 nipple drinkers and 1 feeder under the same environmental condition. The hens were raised at the temperature between 23°C and 26 °C; the relative humidity was 60 to 70%, and there was 16 h of illumination per day (12–20 lux). The birds were allowed ad libitum consumption of feed and water throughout the entire experimental period. This period included a 1-wk adjustment period and a 9-wk experimental stage.

Chemical Analyses in the Feeds

The actual concentrations of Pb in the 4 groups were analyzed by flame atomic absorption spectrophotometry (Thermo Jarrell Ash, Wilmington, MA) according to a previous methodology (Kaya and Yaman, 2008). Crude protein, amino acid, and calcium and phosphorus content were analyzed using procedures from the Association of Official Analytical Chemists.

Zootechnical Performance and Egg Quality

Zootechnical performance was gauged by hen-day egg production, feed consumption, and egg weight (**EW**), which were recorded daily. A sensitive scale (XS2002S; Mettler Toledo, Zurich, Switzerland) was used to determine the feed intake and EW to the nearest 0.01 g. Feed conversion ratio (**FCR**) was calculated as follows: FCR = grams of feed intake/grams of egg mass.

At the age of 50 wk, a total of 30 eggs were randomly collected from each group of hens. Thereafter, Haugh units, albumen height, yolk color, and eggshell strength were measured using a digital egg tester (DET-6000; Nabel Co. Ltd., Kyoto, Japan). In addition, eggshell thickness was measured at the blunt and sharp ends and the middle of each egg using a dial gauge micrometer (547-350; Mitutoyo, Kawasaki, Japan), and the average values were used in the statistical analysis. After egg quality measurement, the albumen and yolk were separated and stored at −20 °C to determine the Pb content.

Collection of Ovary Samples

After the rearing experiment, 24 hens from each group were randomly chosen and euthanized after 24 h of feed withdrawal. The birds were quickly dissected, and their ovaries were removed from their abdominal cavities. Thereafter, the ovaries were separated into four parts. Three ovary parts were frozen in liquid nitrogen immediately and then were stored at −80 °C to measure the accumulation of Pb, oxidative stress biomarkers, and mRNA expression. One part of each ovary was fixed in 4% paraformaldehyde for 24 h to determinate the follicular atresia rate.

Determination of Lead Deposition

After egg quality was determined, the eggs in each group were randomly selected and divided into albumen and yolk. The deposition of Pb in the albumen and yolk was evaluated by flame atomic absorption spectrophotometry (Thermo Jarrell Ash) according to a previous methodology (Kaya and Yaman, 2008). The results were recorded as mg/kg wet weight.

Similarly, flame atomic absorption spectrophotometry was used to determinate the accumulation of Pb in the

Table 1. Composition and nutrient content in the basal diet.

Ingredients	%	Analyzed nutrient content	%, unless noted
Corn	62.00	Metabolizable energy, MJ/kg	10.91
Soybean meal (44.20% CP)	22.00	Crude protein	15.51
Wheat bran	3.00	Lysine	0.78
Limestone	8.00	Methionine	0.34
Calcium phosphate	1.25	Tryptophan	0.16
Sodium chloride	0.40	Total phosphorus	0.48
Premix	3.35	Nonphytate phosphorus	0.37
Total	100.00	Calcium	3.52
		Pb, mg/kg	3.20

The premix provided the following per kilogram of diet: vitamin A, 7,000 IU; vitamin D_3, 2,500 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; choline chloride, 400 mg; Cu, 20 mg; Fe, 70 mg; Mn, 100 mg; Zn, 70 mg; I, 0.4 mg; Se, 0.5 mg.
Abbreviation: Pb, plumbum.

ovaries of laying hens (Kaya and Yaman, 2008). The results are presented as mg/g wet weight.

Determination of the Follicular Atresia Rate

Ovarian samples that were fixed in 4% paraformaldehyde were trimmed and embedded in Paraplast Plus resign (Sigma, Darmstadt, Germany). Thereafter, the ovarian samples were sectioned using a microtome (5-μm-thick section) and stained with hematoxylin and eosin for histological observation under an optical microscope (Olympus America, Melville, NY). Microscopically, 3 glides were randomly chosen from each ovary slide, and the mean values were used in the statistical analysis. The sliding distance between 2 glides was more than 200 nm to avoid counting the number of follicles repeatedly. The number of atretic follicles and normal follicles was counted on each ovary slide, respectively. The histological criteria for normal follicles and follicular atresia were used in reference to a previously published methodology (Gupta et al., 1988). Follicular atresia rate was calculated as follows: follicular atresia rate (%) = number of atretic follicles/number of total follicles × 100. In this equation, the number of total follicles means the number of atretic follicles and the number of normal follicles.

Lipid Peroxidation and Antioxidant Enzyme Activities

The antioxidant activities in the ovaries of laying hens were measured using detection kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) for the enzymes SOD, CAT, glutathione reductase (**GR**), and GSH-Px. In addition, the GSH and MDA contents were also measured using analysis kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocols.

Real-time Quantitative PCR

The total RNA in the ovary samples was extracted using an RNAiso kit (Aidlab, Beijing, China) according to the manufacturer's instructions. The concentration of each extracted RNA sample was measured using a Nanodrop Spectrophotometer (ND-2000; Thermo Fisher Gene Company Ltd., Waltham, MA), and the integrity of the RNA was verified by denatured RNA electrophoresis. The cDNA was synthesized from the total RNA using a PrimeScript RT reagent kit (TaKaRa, Dalian, China) with an oligo dT-adaptor primer according to the manufacturer's instructions. The mRNA abundances of copper–zinc superoxide dismutase (***CuZn-SOD***), manganese superoxide dismutase (***MnSOD***), *CAT*, *GR*, *GSH-Px*, *Nrf2*, *Keap1*, and *β-actin* genes were determined using a CFX96 Touch Sequence Detection System (Bio-Rad, Hercules, CA). The PCRs were each performed using a total volume of 10 μL of iTaq Universal SYBR Green Supermix (2 × 5 μL; Bio-Rad, Hercules, CA), cDNA (1 μL), forward and reverse primers for each gene, and RNase-free dH_2O. The sequences of the real PCR-specific primers are shown in Table 2. The PCR program was set at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 60 °C for 10 s, and 72 °C for 30 s. The efficiencies of the real-time PCR primers were calculated from standard curves. The standard curves were measured using the pooled samples. Specificity of the amplification was examined by melting curve analysis at the end of PCR run. Specificity of the product was verified by running the samples on 1.2% agarose gel, excising for purification using a DNA purification kit (TaKaRa, Dalian, China). The Ct value of *β-actin* <0.5 was considered to be the endogenous control. The $2^{-\Delta\Delta Ct}$ ($\Delta\Delta Ct$ = ΔCt of the target gene − ΔCt of the *β-actin* gene) method was used to collect the data of average gene expression relative to the endogenous control for each sample (Livak and Schmittgen, 2001).

Statistical Analyses

The data were analyzed by one-way ANOVA using SPSS 20.0 software (IBM Corporation, New York, NY), and they are presented as means with their pooled standard errors. The differences in all the groups were determined using Tukey's multiple range tests and considered significant when $P < 0.05$. The orthogonal polynomials for linear and quadratic effects were tested and considered significant at $P < 0.05$.

Table 2. Sequences of real-time PCR–specific primers.

Gene	Primer sequences	PCR product (bp)	Accession number
CuZnSOD	Forward: 5′-CACTGCATCATTGGCCGTACCA-3′ Reverse: 5′-GCTTGCACACGGAAGAGCAAGT-3′	223	NM_205064.1
MnSOD	Forward: 5′-CACTCTTCCTGACCTGCCTTAC-3′ Reverse: 5′-TAGACGTCCCTGCTCCTTATTA-3′	399	NM_204211.1
GSH-Px	Forward: 5′-GCTGTTCGCCTTCCTGAGAG-3′ Reverse: 5′-GTTCCAGGAGACGTCGTTGC-3′	118	NM_001277853.1
CAT	Forward: 5′-TGGCGGTAGGAGTCTGGTCT-3′ Reverse: 5′-GTCCCGTCCGTCAGCCATTT-3′	112	NM_001031215.1
GR	Forward: 5′-ACGGCTCCTCACATCCTCATT-3′ Reverse: 5′-CCAGGTCGAAGAACCCATCAC-3′	109	XM_004943130.1
Nrf2	Forward: 5′-ATCACCTCTTCTGCACCGAA-3′ Reverse: 5′-GCTTTCTCCCGCTCTTTCTG-3′	258	NM_205117.1
Keap1	Forward: 5′-TGCCCCTGTGGTCAAAGTG-3′ Reverse: 5′-GGTTCGGTTACCGTCCTGC-3′	104	XM_015274015.1
β-actin	Forward: 5′-AGCGAACGCCCCCAAAGTTCT-3′ Reverse: 5′-AGCTGGGCTGTTGCCTTCACA-3′	139	NM_205518.1

Abbreviations: *CAT*, catalase; *CuZnSOD*, copper–zinc superoxide dismutase; *GR*, glutathione reductase; *GSH-Px*, glutathione peroxidase; *Keap1*, Kelch-like ECH-associated protein 1; *MnSOD*, manganese superoxide dismutase; *Nrf2*, nuclear factor erythroid 2–related factor 2.

RESULTS

Effect of Dietary Lead Nitrate on the Zootechnical Performance of Laying Hens

The effect of dietary lead nitrate on the zootechnical performance of the hens is shown in Table 3. Both the hen-day egg production ($P < 0.01$) and EW ($P < 0.01$) were significant decreased linearly as the dietary Pb concentration increased from 3.20 to 93.20 mg/kg. There was a significantly linear increase in the FCR as the dietary Pb concentration increased from 3.20 to 93.20 mg/kg ($P < 0.01$). In addition, there was no significant difference in feed intake in all the groups.

Effect of Dietary Lead Nitrate on Egg Quality and Accumulation of Pb in Eggs

The effect of the dietary lead nitrate on egg quality of laying hens is shown in Table 4. Haugh units ($P < 0.01$), albumen height ($P < 0.01$), eggshell thickness ($P < 0.01$), and eggshell strength ($P < 0.01$) were significantly linearly decreased as the Pb concentration increased from 3.20 to 93.20 mg/kg in the diet. In addition, the yolk color showed no significant difference in all the groups.

The effect of dietary lead nitrate on the accumulation of Pb in the eggs is presented in Table 5. There was a significantly linear increase in the accumulation of Pb in the albumen ($P < 0.01$) and yolk ($P < 0.01$) as the

Table 3. Effect of lead nitrate on zootechnical performance of laying hens.[1]

	Dietary Pb concentration (mg/kg)						Contrast *P*-value	
Item[2]	3.20	33.20	63.20	93.20	*P*-value	SEM	L	Q
EP, %	84.22^{a}	81.83^{b}	81.74^{b}	80.38^{b}	<0.01	0.59	<0.01	0.23
EW, g	62.77^{a}	60.96^{b}	59.94^{b}	58.10^{c}	<0.01	0.43	<0.01	0.96
Feed intake, g/day per bird	127.42	126.80	128.27	126.66	0.46	1.20	0.76	0.39
FCR, g of feed/g of egg	2.03^{b}	2.08^{a,b}	2.14^{a,b}	2.18^{a}	0.03	0.05	<0.01	0.85

Abbreviations: EP, hen-day egg production; EW, egg weight; FCR, feed conversion ratio; Pb, plumbum.

[1]Means within each row with different superscripts were significantly different ($P < 0.05$). SEM: standard error of the mean by ANOVA (n = 6); L: linear; Q: quadratic.

[2]Regression equation of EP, y = 84.943–1.162x, R^2 = 0.606; regression equation of EW, y = 64.202–1.504x, R^2 = 0.852; regression equation of FCR, y = 1.976 + 0.052x, R^2 = 0.355.

Table 4. Effect of lead nitrate on egg quality of laying hens.[1]

	Dietary Pb concentration (mg/kg)						Contrast *P*-value	
Item[2]	3.20	33.20	63.20	93.20	*P*-value	SEM	L	Q
Haugh units	89.07^{a}	82.53^{b}	79.12^{b,c}	77.71^{c}	<0.01	1.30	<0.01	0.01
Albumen height, mm	8.26^{a}	7.56^{b}	7.08^{c}	6.43^{d}	<0.01	0.13	<0.01	0.84
Yolk color[3]	7.35	7.09	6.86	7.03	0.17	0.21	0.09	0.18
Eggshell thickness, mm	0.38^{a}	0.37^{a}	0.33^{b}	0.31^{b}	<0.01	0.01	<0.01	0.37
Eggshell strengthen, kgf/m^2	4.17^{a}	3.80^{b}	3.35^{c}	3.16^{c}	<0.01	0.09	<0.01	0.17

[1]Means within each row with different superscripts were significantly different ($P < 0.05$). SEM: standard error of the mean by ANOVA (n = 6); kgf/m^2: kilogram-force/m^2; L: linear; Q: quadratic.

[2]Regression equation of Haugh units, y = 89.654–3.355x, R^2 = 0.554; regression equation of albumen height, y = 8.832–0.599x, R^2 = 0.906; regression equation of eggshell thickness, y = 0.404–0.023x, R^2 = 0.754; regression equation of eggshell strengthen, y = 4.493–0.349x, R^2 = 0.874.

[3]The yolk color is one of the important indexes of egg quality.

Pb concentration increased from 3.20 to 93.20 mg/kg in the diet. In addition, the percentage of Pb in the albumen was a stepwise increase, whereas the percentage of Pb in the yolk was a stepwise decrease.

Effect of Dietary Lead Nitrate on the Follicular Atresia Rate and Accumulation of Pb in the Ovaries of Laying Hens

The effect of dietary lead nitrate on the follicular atresia rate in laying hens is shown in Figure 1A. As the dietary Pb concentration increased from 3.20 to 93.20 mg/kg in the diet, the follicular atresia rate was significantly linearly increased ($P < 0.01$). As shown in Figure 1B, there was a significantly linear increase in the accumulation of Pb in the ovaries of laying hens ($P < 0.01$). Therefore, there was a positive correlation between the accumulation of Pb in the ovaries and the follicular atresia rate (r = 0.952; $P < 0.01$) (Figure 1D).

Effect of Dietary Lead Nitrate on Oxidative Stress Biomarkers in the Ovaries of Laying Hens

The effect of dietary lead nitrate on oxidative stress biomarkers in laying hens is shown in Table 6. The SOD ($P < 0.01$) and GSH-Px ($P < 0.01$) activities and the GSH ($P < 0.01$) contents were significantly decreased quadratically as the Pb concentration increased from 3.20 to 93.20 mg/kg in the diet. Similarly, as the Pb concentration increased from 3.20 to 93.20 mg/kg in the diet, there were significantly linear decreases in the activities of CAT ($P < 0.01$) and GR ($P < 0.01$), whereas the MDA content was significantly increased linearly ($P < 0.01$).

Effect of Dietary Lead Nitrate on Gene Expressions of Antioxidant Enzymes and Nrf2–Keap1 Molecules in the Ovaries of Laying Hens

The effects of dietary lead nitrate on the gene expression of the antioxidant enzymes and Nrf2–Keap1 molecules in laying hens are presented in Table 7. The gene expressions of *CuZnSOD* ($P < 0.01$), *CAT* ($P < 0.01$), and *GR* ($P < 0.01$) were significant decreased linearly as the Pb concentration increased from 3.20 to 93.20 mg/kg in the diet. In addition, there were significantly quadratic decreases in the mRNA expressions of *GSH-Px* ($P < 0.01$) and *Nrf2* ($P < 0.01$). By way of contrast, the Keap1 gene expression was significantly linearly increased ($P < 0.01$) as the Pb concentration increased from 3.20 to 93.20 mg/kg in the diet. In addition, the MnSOD gene expression was not significant in any of the groups.

DISCUSSION

Lead is a hypertoxic and common environmental contaminant. Lead induces a number of toxicoses in

Table 5. Effect of lead nitrate on accumulation of Pb in the egg of laying hens.[1]

	Dietary Pb concentration (mg/kg)						Contrast *P*-value	
Item[2]	3.20	33.20	63.20	93.20	*P*-value	SEM	L	Q
Albumen, mg/kg	0.20^{c}	0.27^{c}	0.51^{b}	0.72^{a}	<0.01	0.03	<0.01	0.02
Yolk, mg/kg	0.31^{d}	0.61^{c}	0.90^{b}	1.20^{a}	<0.01	0.04	<0.01	0.96
Albumen, %	38.99^{a}	30.50^{b}	36.32^{a,b}	37.63^{a}	<0.01	2.28	0.81	<0.01
Yolk, %	61.01^{b}	69.50^{a}	63.68^{a,b}	62.37^{b}	<0.01	2.28	0.81	<0.01

Abbreviation: Pb, plumbum.

[1]Means within each row with different superscripts were significantly different ($P < 0.05$). SEM: standard error of the mean by ANOVA (n = 6); L: linear; Q: quadratic. Accumulation of Pb in egg = accumulation of Pb in albumen + accumulation of Pb in yolk. Albumen, % = accumulation of Pb in albumen/accumulation of Pb in egg. Yolk, % = accumulation of Pb in yolk/accumulation of Pb in egg.

[2]Regression equation of the accumulation of Pb in albumen, y = −0.027 + 0.181x, R^2 = 0.923; regression equation of the accumulation of Pb in yolk, y = 0.014 + 0.296x, R^2 = 0.968.

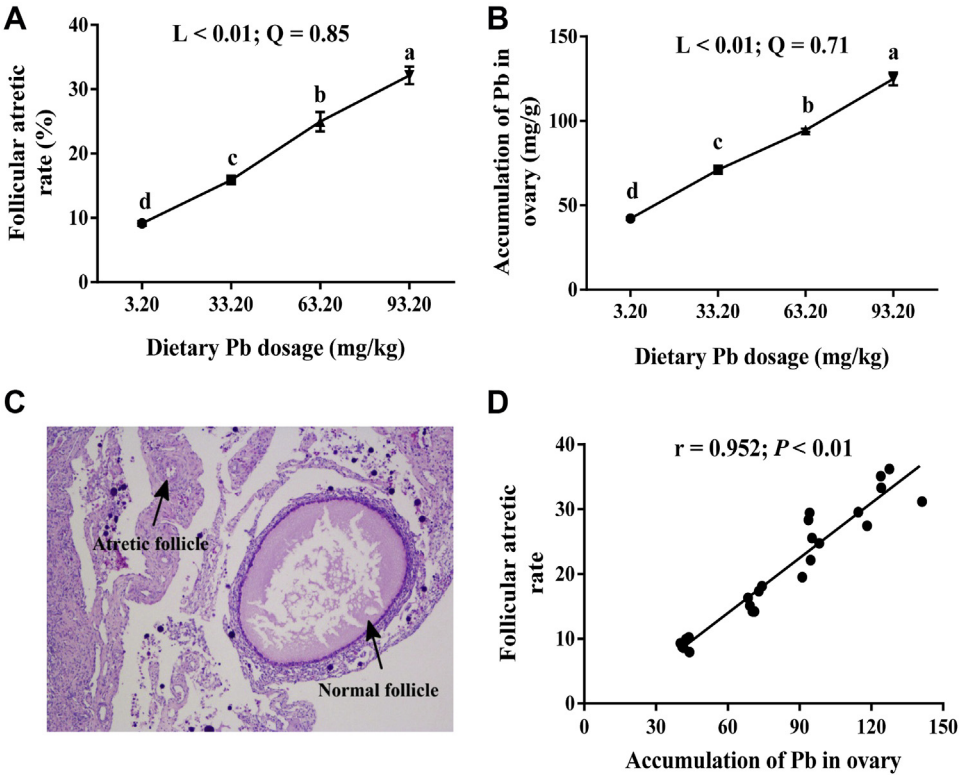

Figure 1. Effect of dietary lead nitrate on accumulation of Pb in the ovary of laying hens and follicular atresia rate in laying hens. (A) Follicular atresia rate in laying hens. (B) Accumulation of Pb in the ovary of laying hens. (C) Representative histological observation of normal follicular and atresia follicular (40×). (D) Pearson's correlation analysis between the follicular atresia rate and accumulation of Pb in the ovary. Standard error of the mean by ANOVA (n = 6). Mean values with different superscripts were different significantly among 4 groups ($P < 0.05$). Regression equation of the accumulation of Pb in the ovary, y = 15.231 + 27.176x, R^2 = 0.976; regression equation of follicular atresia rate, y = 1.016 + 7.808x, R^2 = 0.925. Abbreviations: L, linear; Pb, plumbum; Q, quadratic.

organs and systems, including cardiotoxicity, central and peripheral nervous system intoxication, nephrotoxicity, hepatotoxicity, and reproductive toxicity (Todd et al., 1996). The reproductive system is always one of the most important targets of Pb poisoning. Previous studies have shown that Pb exposure induces a number of deleterious effects in the reproductive system, including plasma testosterone reduction, premature acrosome reaction, reduced zona-intact oocyte-penetrating capability, and spermatogenesis damage (Batra et al., 1998; Hsu et al., 1998). In this study, we found that the zootechnical performance was linearly decreased as the dietary Pb concentration increased. Vodela et al. (1997) reported that there was a linear negative relationship between the reproductive performance of broiler breeders and the increasing concentration of Pb in the drinking water, which was in accordance with the result of this study. In the study, the percentage of Pb in the albumen was a stepwise increase, whereas the percentage of Pb in the yolk was a stepwise decrease. It appears that the yolk (egg cell) counter-regulated in the higher dosages, shifting more

Table 6. Effect of lead nitrate on oxidative stress biomarkers in the ovary of laying hens.[1]

	Dietary Pb concentration (mg/kg)						Contrast *P*-value	
Item[2]	3.20	33.20	63.20	93.20	*P*-value	SEM	L	Q
SOD, U/mg protein	169.42^{a}	110.49^{b}	98.57^{b,c}	91.17^{c}	<0.01	4.29	0.01	<0.01
CAT, U/mg protein	7.51^{a}	4.36^{b}	3.45^{c}	2.07^{d}	<0.01	0.18	<0.01	0.01
GR, U/g protein	6.81^{a}	2.66^{b}	2.17^{c}	1.86^{d}	<0.01	0.09	<0.01	0.01
GSH-Px, U/mg protein	16.97^{a}	13.18^{b}	12.48^{c}	11.81^{d}	<0.01	0.50	0.01	<0.01
MDA, nmol/mg protein	2.56^{d}	3.80^{c}	8.96^{b}	12.17^{a}	<0.01	0.39	<0.01	0.02
GSH, μM/L	117.00^{a}	64.24^{b}	63.36^{b}	52.11^{c}	<0.01	3.02	0.01	<0.01

Abbreviations: CAT, catalase; GR, glutathione reductase; GSH, glutathione; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; Pb, plumbum; SOD, superoxide dismutase.

[1]Means within each row with different superscripts were significantly different ($P < 0.05$). SEM: standard error of the mean by ANOVA (n = 6); L: linear; Q: quadratic.

[2]Regression equation of SOD, y = 243.495–89.079x + 12.882x^2, R^2 = 0.931; regression equation of CAT, y = 8.653–1.723x, R^2 = 0.911; regression equation of GR, y = 7.215–1.536x, R^2 = 0.729; regression equation of GSH-Px, y = 21.553–5.512x + 0.778x^2, R^2 = 0.841; regression equation of MDA, y = −1.632 + 3.401x, R^2 = 0.932; regression equation of GSH, y = 174.958–71.445x + 10.378x^2, R^2 = 0.891.

Table 7. Effect of lead nitrate on gene expressions of antioxidant enzymes and Nrf2–Keap1 molecules in the ovary of laying hens.[1]

	Dietary Pb concentration (mg/kg)						Contrast *P*-value	
Item[2]	3.20	33.20	63.20	93.20	*P*-value	SEM	L	Q
CuZnSOD	0.99[a]	0.80[b]	0.55[c]	0.37[d]	<0.01	0.03	<0.01	0.76
MnSOD	0.96	0.91	0.99	1.01	0.68	0.08	0.39	0.57
CAT	1.00[a]	0.75[b]	0.51[c]	0.33[d]	<0.01	0.05	<0.01	0.29
GR	1.11[a]	0.79[b]	0.48[c]	0.46[c]	<0.01	0.06	<0.01	0.03
GSH-Px	1.09[a]	0.53[b]	0.56[b]	0.45[b]	<0.01	0.06	0.01	<0.01
Nrf2	1.37[a]	0.78[b]	0.54[c]	0.39[d]	<0.01	0.05	0.01	<0.01
Keap1	0.75[d]	1.22[c]	1.63[b]	2.16[a]	<0.01	0.11	<0.01	0.73

Abbreviations: *CAT*, catalase; *CuZnSOD*, copper–zinc superoxide dismutase; *GR*, glutathione reductase; *GSH-Px*, glutathione peroxidase; *Keap1*, Kelch-like ECH-associated protein 1; *MnSOD*, manganese superoxide dismutase; *Nrf2*, nuclear factor erythroid 2–related factor 2; Pb, plumbum.

[1]Means within each row with different superscripts were significantly different ($P < 0.05$). SEM: standard error of the mean by ANOVA (n = 6); L: linear; Q: quadratic.

[2]Regression equation of *CuZnSOD*, y = 1.207–0.213x, R^2 = 0.948; regression equation of *CAT*, y = 1.213–0.227x, R^2 = 0.899; regression equation of *GR*, y = 1.280–0.228x, R^2 = 0.806; regression equation of *GSH-Px*, y = 1.695–0.755x + 0.113x^2, R^2 = 0.790; regression equation of *Nrf2*, y = 2.110–0.862x + 0.109x^2, R^2 = 0.952; regression equation of *Keap1*, y = 0.276 + 0.465x, R^2 = 0.902.

of the Pb into albumen and out of the cell. This may indicate compensation reactions to the threat of Pb intoxication in laying hens. Haugh units, albumen height, yolk color, eggshell thickness, and egg strength are known as important indexes for measuring the egg quality (Silversides, 1994). A previous investigation showed that egg quality of broiler breeders might be affected by the presence of lead in drinking water (Vodela et al., 1997). In the present study, Haugh units, albumen height, eggshell thickness, and eggshell strength were linearly reduced as the dietary Pb concentration increased. Besides, a previous study showed that eggshell performance was closely related to the synthesis of functional peptides in laying hens (Clunies et al., 1992; Yuan et al., 2013). Hence, we inferred that a reduced redox capacity of the layer organism affected the synthesis of functional peptides. In addition, there was no relationship between dietary Pb exposure and yolk color in this study. As we know, the carotenoid supply could affect yolk color in laying hens. The previous study showed that carotenoid uptake is mediated by the apical transporter scavenger receptor class B type I, and carotenoid efflux occurs exclusively via its secretion in chylomicron. Thus, we speculated that the class B type I might not be affected by Pb exposure in laying hens.

The reproductive system in female animals is susceptible to disturbance after Pb exposure (Winder, 1993). The ovary is an important vital organ in the reproductive system, and it is vulnerable to damage from lead. In the breeding industry, ovary performance is extraordinarily important for laying hens. In this study, lead accumulation in the ovaries of laying hens was linearly increased as dietary Pb concentration increased. In addition, the follicular atresia rate was positively correlated with the accumulation of Pb in the hen ovaries, implying that ovary lead accumulation might affect the zootechnical performance by enhancing the follicular atresia rate in laying hens. A previous study reported that dysfunction in folliculogenesis, with fewer primordial follicles and an increase in atretic antral follicles, was caused by a low concentration of lead in the ovaries of mice (Taupeau et al., 2001). Another study found that in comparison with other visceral organs, lead accumulated the most in the ovaries of sows, and the sows' reproductive performance was correspondingly damaged owing to their Pb exposure (Phillps et al., 2003).

Substantial evidence has suggested that the ovarian dysfunction mechanism of Pb-induced toxicity was correlated with oxidative stress by suppressing the antioxidant defense system (Behrman et al., 2001). Intracellular lipid peroxidation can be monitored by following the MDA content. In addition, the intracellular GSH is an important nonenzymatic system for defending against oxidative stress (Finkel and Holbrook, 2000). In the present study, dietary lead exposure induced an enhancement in the MDA content linearly and reduced the GSH level quadratically in the ovaries of laying hens, implying that ovarian intracellular lipid peroxidation was activated in response to ovarian injury. Similar results reported that lead significantly enhanced the intracellular MDA content and reduced the intracellular GSH level in Chinese hamster ovary cells, which was consistent with this study (Ercal et al., 1996). In addition, the enzymatic antioxidant system also played vital roles in attenuating the ovarian oxidative stress induced by lead exposure, and it included SOD, CAT, GR, GSH-Px, and other enzymes. In this study, we found that there were significantly linear decreases in the CAT and GR activities, and there were significantly quadratic decreases in the SOD and GSH-Px activities as the Pb concentration increased from 3.20 to 93.20 mg/kg in the diet. A previous study evaluated the oxidative stress in seminal plasma and spermatozoa after high levels of lead exposure. The results showed that lead exposure could significantly reduce the activities of antioxidant enzymes and the GSH level, which was in accordance with this study (Kiziler et al., 2007). Interestingly, a previous study also reported that

antioxidant enzymes played a protective role against mercury-induced oxidative stress and enhanced the activities of antioxidant enzymes in the reproductive systems of zebra fish, which was actually not inconsistent with this study. At the first stage of oxidative stress, the antioxidant system would enhance its activities to neutralize oxidative damage, but when the antioxidant system could not eliminate the excess intracellular reactive oxygen species, it would in turn reduce the activities of the antioxidant enzymes or even weaken the antioxidant defense system, which was in accordance with a previous report (Morcillo et al., 2016). The activities of antioxidant enzymes can be regulated by the relevant gene expressions because of their protein properties (Tiedge et al., 1997). In the present study, except for *MnSOD* gene expression, the changes in *CuZnSOD*, *CAT*, *GR*, and *GSH-Px* mRNA expression were in accordance with the relevant enzyme activities, implying that these activities might be regulated by Pb exposure at the transcriptional level. In addition, despite the fact that the organism would be in need for these antioxidant enzymes, the lead exposure might have affected the protein metabolism, including the synthesis of antioxidant enzymes. Hence, there was a simultaneous decrease in activity and transcription of these enzymes in this study, which was in accordance with a previous study (Ma et al., 2018b). However, there was no correlation between *MnSOD* gene expression and SOD activities in the ovaries of laying hens. A possible reason for the mismatched relationship was that the SOD enzymes had more than 2 types of isoenzymes, and the SOD activities are equal to those of all the isoenzymes (Nam et al., 2005). Thus, as only one of all the isoenzymes, the *MnSOD* gene might not transcribe the entire SOD in ovaries. Furthermore, CuZnSOD is a cytoplasmic enzyme, whereas MnSOD enzyme is associated with mitochondria (Flanagan et al., 2002). We speculated that Pb exposure might primarily affect the cytoplasmic SOD activity and could not disturb the synthesis of mitochondrial SOD in the ovary of laying hens.

There are several known protective mechanisms against oxidative stress (Li et al., 2012; Ma et al., 2018b). The redox-sensitive Nrf2–Keap1 pathway served as a key signaling pathway in maintaining redox homeostasis and mobilizing the cellular antioxidant defenses. In the present study, lead exposure could significantly reduce intracellular *Nrf2* gene expression. In addition, there were positive relationships between *Nrf2* gene expression and the gene expression of antioxidant enzymes. These trends indicated that Nrf2 could regulate the downstream gene expressions in the ovaries of laying hens. In addition, a negative correlation occurred between *Keap1* and *Nrf2* mRNA expression or *Keap1* mRNA expression and the gene expression of the antioxidant enzymes in this study. We speculated that the upregulation of the intracellular Keap1 level could promote cytoplasmic Nrf2 degradation, and then, it inhibited the mRNA expression of antioxidant enzymes downstream after the ovaries of laying hens were exposed to Pb. A previous study reported that the Nrf2–Keap1 pathway was suppressed in the ovaries of laying hens after mercury exposure, which was in accordance with this study (Ma et al., 2018b). Nevertheless, another study showed that a defense mechanism would be activated through the Nrf2–Keap1 signal pathway after heavy metal exposure in fish (Zeng et al., 2016). In fact, the previous investigation was not inconsistent with the result in this study. At the early stage of oxidative stress, the Nrf2–Keap1 pathway was activated to defend against the oxidative damage, but when oxidative damage occurred at the continuous high Pb dose, the Nrf2–Keap1 pathway was then suppressed (Kensler et al., 2007). This study provides some sound evidence for antioxidant defense under Pb exposure in the ovary of laying hens for the first time. In future, a more finely graded dose–response study will be carried out to find the minimum Pb dosages to induce negative effects.

In summary, dietary lead exposure could significantly and linearly reduce the zootechnical performance and egg quality. Furthermore, the possible reason for the reduced zootechnical performance is that dietary lead exposure increased the follicular atresia rate in the ovaries of laying hens. In addition, the dietary Pb exposure induced oxidative stress and reduced antioxidant enzyme activities by suppressing the Nrf2–Keap1 signal pathway in the ovaries of laying hens.

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REFERENCES

Ahamed, M., and M. K. J. Siddiqui. 2007. Low level lead exposure and oxidative stress: Current opinions. Clin. Chim. Acta 383:57–64.

Batra, N., B. Nehru, and M. P. Bansal. 1998. The effect of zinc supplementation on the effects of lead on the rat testis. Reprod. Toxicol. 12:535–540.

Behrman, H. R., P. H. Kodaman, S. L. Preston, and S. Gao. 2001. Oxidative stress and the ovary. J. Soc. Gynecol. Investiq. 8:40–42.

Caban, G., and J. B. Rasmussen. 1994. Modelling food chain structure and contaminant bioaccumulation using stable nitrogen isotopes. Nature 372:255–257.

Clunies, M., D. Parks, and S. Leeson. 1992. Calcium and phosphorus metabolism and eggshell formation of hens fed different amounts of calcium. Poult. Sci. 71:482–489.

Ercal, N., P. Treeratphan, P. Lutz, T. C. Hammond, and R. H. Matthews. 1996. N-acetylcysteine protects Chinese hamster ovary (CHO) cells from lead-induced oxidative stress. Toxicology 15:57–64.

Farmer, A. A., and A. M. Farmer. 2000. Concentrations of cadmium, lead and zinc in livestock feed and organs around a metal production centre in eastern Kazakhstan. Sci. Total Environ. 257:53–60.

Finkel, T., and N. J. Holbrook. 2000. Oxidants, oxidative stress and the biology of aging. Nature 408:239–247.

Flanagan, S. W., R. D. Anderson, M. A. Ross, and L. W. Oberley. 2002. Overexpression of manganese superoxide dismutase attenuates neuronal death in human cells expressing

mutant (G37R) Cu/Zn-superoxide dismutase. J. Neurochem. 81:170–177.
Gupta, S. K., A. B. Gilbert, and M. A. Walker. 1988. Histological study of follicular atresia in the ovary of the domestic hen (Gallus domesticus). J. Reprod. Fertil. 82:219–225.
Hsu, P. C., C. C. Hsu, M. Y. Liu, L. Y. Chen, and Y. L. Guo. 1998. Lead-induced changes in spermatozoa function and metabolism. J. Toxicol. Environ. Health Sci. 55:45–64.
Jaishankar, M., T. Tseten, N. Anbalagan, B. B. Mathew, and K. N. Beeregowda. 2014. Toxicity, mechanism and health effects of some heavy metals. Interdiscip. Toxicol. 7:60–72.
Kaya, G., and M. Yaman. 2008. Online preconcentration for the determination of lead, cadmium and copper by slotted tube atom trap (STAT)-flame atomic absorption spectrometry. Talanta 75:1127–1133.
Kensler, T. W., N. Wakabayashi, and S. Biswal. 2007. Cell survival responses to environmental stress via the Keap1-Nrf2-ARE pathway. Annu. Rev. Pharmacol. Toxicol. 47:89–116.
Kiziler, A. R., B. Aydemir, I. Onaran, B. Alici, H. Ozkara, T. Gulyasar, and M. C. Akyolcu. 2007. High levels of cadmium and lead in seminal fluid and blood of smoking men are associated with high oxidative stress and damage in infertile subjects. Biol. Trace Elem. Res. 120:82–91.
Kwak, M. K., K. Itoh, M. Yamamoto, T. R. Sutter, and T. W. Kensler. 2001. Role of transcription factor Nrf2 in the induction of hepatic phase 2 and antioxidative enzymes in vivo by the cancer chemoprotective agent, 3H-1, 2-dithiole-3-thione. Mol. Med. 7:135–145.
Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25:402–408.
Li, X. X., Y. G. Yang, Y. Q. Lu, S. S. Lu, M. Zhang, H. I. Yao, L. J. Meng, and K. H. Lu. 2012. Protective effects of melatonin against oxidative stress in flow cytometry-sorted buffalo sperm. Reprod. Domest. Anim. 47:299–307.
Lockitch, G. 1993. Perspectives on lead toxicity. Clin. Biochem. 26:291–296.
Long, M., Y. Liu, Y. Cao, N. Wang, M. Dang, and J. B. He. 2016. Proanthocyanidins attenuation of chronic lead-induced liver oxidative damage in Kunming mice via the Nrf2/ARE pathway. Nutrients 8:656.
Ma, Y., Y. Z. Shi, L. L. Li, C. Xie, and X. T. Zou. 2018a. Toxicology effects of mercury chloride on laying performance, egg quality, serum biochemistry, and histopathology of liver and kidney in laying hens. Biol. Trace Elem. Res. 185:465–474.
Ma, Y., M. K. Zhu, L. P. Miao, X. Y. Zhang, X. Y. Dong, and X. T. Zou. 2018b. Mercuric chloride induced ovarian oxidative stress by suppressing Nrf2-Keap1 signal pathway and its downstream genes in laying hens. Biol. Trace Elem. Res. 185:186–196.
Morcillo, P., M. Á. Esteban, and A. Cuesta. 2016. Heavy metals produce toxicity, oxidative stress and apoptosis in the marine teleost fish SAF-1 cell line. Chemosphere 144:225–233.
Nakade, U. P., S. K. Garq, A. Sharma, S. Choudhury, R. S. Yadav, K. Gupta, and N. Sood. 2015. Lead-induced adverse effects on the reproductive system of rats with particular reference to histopathological changes in uterus. Indian J. Pharacol. 47:22–26.
Nam, Y. K., Y. S. Cho, B. N. Chio, K. H. Kim, S. K. Kim, and D. S. Kim. 2005. Alternation of antioxidant enzymes at the mRNA level during shortterm starvation of rock-bream Oplegnathus fasciatus. Fish Sci. 71:1385–1387.
Papanikolaou, N. C., E. G. Hatzidaki, S. Belivanis, G. N. Tzanakakis, and A. M. Tsatsakis. 2005. Lead toxicity update. A brief review. Med. Sci. Monit. 11:RA329–RA336.
Patrick, L. 2006. Lead Toxicity Part II: the role of free radical damage and the use of antioxidants in the pathology and treatment of lead toxicity. Altern. Med. Rev. 11:114–127.
Phillps, C., Z. Győri, and B. Kovács. 2003. The effect of adding cadmium and lead alone or in combination to the diet of pigs on their growth, carcase composition and reproduction. J. Sci. Food Agric. 83:1357–1365.
Pinon-Lataillade, G., A. Thoreux-Manlay, H. Coffiqny, G. Monchaux, R. Masse, and J. C. Soufir. 1993. Effect of ingestion and inhalation of lead on the reproductive system and fertility of adult male rats and their progeny. Hum. Exp. Toxicol. 12:165–172.
Reglero, M. M., M. A. Taggart, L. Monsalve-González, and R. Mateo. 2009. Heavy metal exposure in large game from a lead mining area: effects on oxidative stress and fatty acid composition in liver. Environ. Pollut. 157:1388–1395.
Silversides, F. G. 1994. The Haugh unit correction for egg weight is not adequate for comparing eggs from chickens of different lines and ages. J. Appl. Poult. Res. 3:120–126.
Taupeau, C., J. Poupon, F. Nomé, and B. Lefèvre. 2001. Lead accumulation in the mouse ovary after treatment-induced follicular atresia. Reprod. Toxicol. 15:385–391.
Tiedge, M., S. Lortz, J. Drinkgern, and S. Lenzen. 1997. Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. Diabetes 46:1733–1742.
Todd, A. C., J. G. Wetmur, J. M. Moline, J. H. Godbold, S. M. Levin, and P. J. Landriqan. 1996. Untraveling the chronic toxicity of lead: an essential priority for environmental health. Environ. Health Perspect. 1:141–146.
Vodela, J. K., S. D. Lenz, J. A. Renden, W. H. McElhenney, and B. W. Kemppainen. 1997. Drinking water contaminations (arsenic, cadmium, lead, benzene, and trichloroethylene). 2. Effects on reproductive performance, egg quality, and embryo toxicity in broiler breeders. Poult. Sci. 76:1493–1500.
Winder, C. 1993. Lead, reproduction and development. Neurotoxicology 14:303–317.
Yuan, C., H. H. Song, Y. J. Jiang, M. M. M. Azzam, S. Zhu, and X. T. Zou. 2013. Effects of lead contamination in feed on laying performance, lead retention of organs and eggs, protein metabolism, and hormone levels of laying hens. J. Appl. Poult. Res. 22:878–884.
Zeng, L., J. L. Zheng, Y. H. Wang, M. Y. Xu, A. Y. Zhu, and C. W. Wu. 2016. The role of Nrf2/Keap1 signaling in inorganic mercury induced oxidative stress in the liver of large yellow croaker *Pseudosciaenacrocea*. Ecotoxicol. Environ. Saf. 132:345–352.
Zhang, D. D. 2006. Mechanistic studies of the Nrf2-Keap1 signaling pathway. Drug Metab. Rev. 38:769–789.