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Bioaccumulation and Toxicity Studies of Lead and Mercury in Laying Hens: Effects on Laying Performance, Blood Metabolites, Egg Quality and Organ Parameters

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This study investigated bioaccumulation and toxicity derived from heavy metals in laying hens. The 160 52-week old laying hens were divided into 5 treatments with 8 replicates of 4 birds per pen. The treatments consisted of the control diet (without heavy metals), control diet with half the available dosage (AD, 5 ppm lead and 0.2 ppm mercury), AD (10 ppm lead and 0.4 ppm mercury), 2-fold AD (20 ppm lead and 0.8 ppm mercury), and 3-fold AD (30 ppm lead and 1.2 ppm mercury), and were provided to the laying hens for 8 weeks. Food and water were provided on an *ad libitum* basis at all times. Body weight and food intake were recorded once every two weeks, and eggs were collected and recorded daily. Two birds from each pen were euthanized to collect blood and organ samples on week 4 and 8. The 3-fold AD diet reduced food intake compared to that of the control and AD diets ($P<0.05$). Hens fed the half AD diet had darker yolk compared to those fed the control and AD diet on week 4 ($P<0.05$). Hens fed the 2- and 3-fold AD diets had increased relative liver weight, blood glutamic pyruvic transaminase and glutamic oxaloacetic transaminase levels ($P<0.05$), while F1 follicle weights decreased on week 4 and 8. No difference was found in egg production rate, egg quality, ovarian follicle, blood metabolites including protein, globulin, albumin, and urea nitrogen throughout the study ($P>0.05$). Heavy metal concentrations in the liver, eggs, and feathers were not detected at both week 4 and 8. Our results indicate that in-feed heavy metals for layer diets up to 30 ppm of lead and 1.2 ppm of mercury brought on hepatic dysfunction increasing blood metabolites that are associated with liver inflammation.

Key words: bioaccumulation, feed, laying hen, lead, mercury, toxicity

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Introduction

Egg-type chickens are susceptible to toxicity of heavy metals often resulting in negative economic impacts, inducing higher mortality, lowering reproductive output, and weakening eggshell strength (Vodela *et al*., 1997; Dauwe *et al*., 2004). For example, lead and mercury are known as reprotoxic substances that can cause destructive effects such as hepatitis and kidney damage when birds are exposed at very low levels (Ibrahim *et al*., 2006). Particularly, when birds are exposed to lead and mercury together, it is believed that the metabolic action of two heavy metals imitates the calcium metabolism to absorb into bone with higher affinity for osteocalcin than calcium, increasing bone turnover and disturbing the calcium metabolic pathway, resulting in hypercalciuria (Dowd *et al*., 2001). In this regard, heavy metals are likely to interfere with laying hens eggshell formation and skeletal metabolism, which are directly associated with their market performance.

With this mind, the legislation on heavy metal concentration in animal diets has been established. For instance, according to EU legislation, mercury (Hg) in feed materials should not exceed 0.1 ppm and lead (Pb) in complete feeds is limited at 5 ppm in a chicken diet. In addition, in the feed safety limits of South Korea, Hg and Pb levels in the complete formulated feed for chicken should be limited under at

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0.4 and 10 ppm, respectively. However, differences in the available dose of lead and mercury among countries have not yet been explored. Therefore, the aim of this study was to evaluate toxicity symptoms of dietary lead and mercury in laying hens from 52 to 60 weeks of age.

Materials and Methods

The protocol of this study was reviewed and approved by the Animal Ethics Committee of Chungnam National University (CNU-00981).

Study Design

The experiment was designed according to the guideline for feed formulation of livestock by the Ministry of Agriculture, Food and Rural Affairs (South Korea). Laying hens were obtained from a commercial layer farm (Icheon-si, Gyeonggi-do, Republic of Korea) and provided a 2-week adjustment period after transportation to overcome any adverse effects on their performance. After the adjustment period, 160 52-week old Lohmann brown laying hens with similar body weights were randomly allocated to one of the five dietary treatments so that each treatment had 8 replications (4 birds per pen) for 8 weeks. The 5 dietary treatments used in this experiment were diets supplemented without heavy metals (control), 5 ppm Pb and 0.2 ppm Hg (half available dose (AD)), 10 ppm Pb and 0.4 ppm Hg (AD), 20 ppm Pb and 0.8 ppm Hg (2-fold AD), or 30 ppm Pb and 1.2 ppm Hg (3-fold AD). Eggs laid and mortality were recorded daily, and body weight and feed intake were measured once every two weeks One hen from each replicate in week 4 and 8 was selected and euthanized to collect blood samples and to measure organ parameters.

Birds, Diets, and Management

Experimental diets were formulated according to the South Korean feed legislation on heavy metal contents. Lead and mercuric chloride ($HgCl_2$) were obtained from Sigma-Aldrich (Product No. 391352 and 215465, respectively) and were added to the diets as top-dressing method. Laying hens were kept in the environmentally controlled poultry house at 23±2℃ under a 13 h Light: 11 h Dark (20 lux) lighting program. All hens were allowed food and water on an *ad libitum* basis. The composition and calculated value of the basal diet are presented in Table 1.

Sample Collection

On week 4 and 8, two birds selected from each replicate were weighed and sacrificed, and then blood samples were collected from the jugular vein into EDTA vacutainers. Blood samples were centrifuged at 3000 rpm for 10 min at 4℃, then plasma was separated and kept at −80℃ for further analysis. Feathers, livers, kidneys, ovaries, and oviducts were dissected from the birds for weighing. After weighing the liver, the middle lobe of the liver was collected into a 2 mL micro tube and kept at −20℃ to analyze the lead and mercury concentrations.

Data Collection

During the experiment, all eggs were collected once a day (09:00 am) and were recorded on a per pen basis. The egg production rate was calculated based on collected data. Egg quality analysis was conducted once every two weeks for 8 weeks. From the dissected ovary, follicles were classified based on size and their numbers were counted. When yellow follicles were over 10 mm in diameter, they were considered as a large yellow follicle, or were otherwise noted as a small yellow follicle (under 10 mm in diameter). The number of large white follicles were counted when the diameter was over 5 mm.

Table 1. **Basal diet**

Ingredients (%)	Contents
Corn	56.17
Wheat bran	3.55
Soybean meal	26.35
Vegetable oil	2.00
Limestone	9.20
Dicalcium Phosphate	1.95
Salt	0.30
Vitamin-mineral premix	0.30
DL-Methionine	0.18
Calculated value	
Metabolizable energy (kcal/kg)	2,800
Crude protein (%)	17.9
Calcium (%)	4.1
Available phosphorous (%)	0.46
Lysine (%)	0.97
Methionine (%)	0.46

[1] Provided per kg of air-dry diet: Vitamin A, 12,000 IU; Vitamin D, 33,000 IU; Vitamin E; 15 mg; Vitamin K, 2 mg; thiamine 2 mg; riboflavin 6 mg; pyridoxine 2 mg; calcium pantothenate 0.03 mg; folic acid 0.2 mg; niacin 45 mg; biotin 0.15 μg; Calcium 0.5%; Cobalt from cobalt sulphate, 0.5 mg; Copper from copper sulphate, 10 mg; Iodine from potassium iodine 0.9 mg; Iron from ferrous sulphate, 80 mg; Manganese from manganous oxide, 80 mg; Selenium from sodium selenite, 0.2 mg; Zinc from zinc oxide, 80 mg.

[2] The values are calculated according to the values of feedstuffs in NRC (1994).

Chemical Analyses

Blood plasma was used to analyze glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), γ-glutamyl transferase (GGT), and urea nitrogen (UN) levels by the enzymatic assay method using an Automatic Analyzer 7180 (Hitachi, Tokyo, Japan). All used kits were purchased from Sekisui Chemical (Tokyo, Japan). Egg, liver, blood, and feather samples were analyzed for concentrations of lead and mercury using an Agilent 7700x quadrupole ICP-MS (Agilent Technologies, Waldbronn, Germany) with a dual mode discrete dynode electron multiplier detector. Eight eggs from each treatment per week were randomly selected and egg length and width were measured using a pair of digital calipers (DC 200, CAS). Egg quality parameters such as shell color, albumin height, Haugh unit, and yolk color were determined using the GCM+ System (Technical Services and Supplies, York, England). Eggshell strength was tested using a TA-XT Plus texture analyzer (Texture Technologies Corp., Scarsdale, NY, USA)

Statistical Analyses

Statistical analysis was performed by SPSS statistics ver. 25 (IBM SPSS Inc., Chicago, IL). Data on growth and laying performance were analyzed on a per pen basis. Data on egg quality was based on individual selected eggs and data on organ parameters and blood metabolites were based on selected laying hens from each replicate. All data were checked for normal distribution and equal variance and then the differences between the treatments were assessed using a one-way analysis of variance (ANOVA) using the general linear model procedure. The comparisons of treatment averages were performed using a Tukey honestly significant difference (HSD) test. Statistical significance was considered to be at $P<0.05$, and $0.05<P<0.10$ was considered a trend.

Results

There was no mortality in the laying hens exposed to dietary heavy metals during the 8 week experimental period.

Growth and Laying Performance

The effect of dietary heavy metal levels on body weight and average daily food intake is presented in Table 2. Body weights and average daily food intake from across the 8 week study period were not affected by dietary heavy metals exposure ($P>0.05$).

The effect of dietary heavy metals on egg production rate is presented in Table 3. There was no effect of different dietary heavy metal levels on the egg production rate ($P>0.05$).

Egg Quality

The effect of external and internal egg qualities by different dietary heavy metal levels are presented in Table 4, of which there were no significant effects ($P>0.05$).

Organ Parameters

The absolute and relative weights of the organs measured are presented in Table 5. Hens fed the control diet had a lighter liver weight relative to body weight on week 4 compared to that of hens fed the 3-fold AD diet ($P=0.024$). Relative spleen weight to body weight on week 4 had a trend for increased spleen weight as dietary heavy metal levels increased ($P=0.081$).

Additionally, absolute liver weight increased on week 8 when hens were exposed to any level of dietary heavy metals compared to that of hens fed the control diet ($P<0.001$), with the heaviest liver weight found in hens fed the 3-fold AD diet. Moreover, relative liver weight on week 8 also increased when hens were fed the half AD and 3-fold AD diets compared to that of hens fed the control diet ($P=0.049$).

The number of ovarian follicles and the weight of F1 follicle are presented in Table 6. The weight of F1 follicles were lighter on week 4 in hens fed the 3-fold AD diet when compared to that of hens fed the control and half AD diets ($P=0.011$); however, no difference was observed on week 8. The number of large yellow, small yellow, and large white follicles did not differ among the treatments.

Table 2. **Body weight and average daily feed intake (g)[1] of layer chickens exposed to different levels of mercury and lead contamination**

Item	Control	Half AD	AD	2-fold AD	3-fold AD	SEM	P-value
Body weight							
Initial	1886.7	1828.8	1861.6	1940.3	1869.8	13.53	0.115
Week 2	1875.1	1827.8	1833.4	1902.4	1853.0	10.05	0.102
Week 4	1923.4	1959.2	1951.0	1888.8	1975.2	13.26	0.268
Week 6	1909.6	1941.6	1934.3	1853.3	1988.7	15.71	0.085
Week 8	1911.7	1985.4	1917.3	1847.3	1988.5	18.05	0.066
Average daily feed intake							
Week 0–2	102.08	102.25	99.03	100.16	103.84	1.037	0.638
Week 2–4	127.76	128.39	126.68	127.55	126.26	0.619	0.838
Week 4–6	130.29	125.86	128.56	117.08	110.33	2.502	0.069
Week 6–8	109.45	116.35	121.61	105.2	119.87	2.752	0.278

[1] $n=8$ per treatment (pen was considered as the experimental unit, 4 birds per pen).
[a–b] Means with different superscripts in the same row were significantly different (Tukey's HSD, $P<0.05$).

Table 3. **Egg production rate (%)[1]**

Item	Control	Half AD	AD	2-fold AD	3-fold AD	SEM	P-value
Week 0–2	67.41	63.39	56.25	57.37	64.29	2.631	0.644
Week 2–4	86.61	87.50	90.85	79.91	91.52	1.829	0.282
Week 4–6	89.59	87.80	90.48	90.48	92.56	1.521	0.916
Week 6–8	84.23	91.67	91.67	86.61	87.20	2.061	0.748

[1] $n=8$ per treatment (pen was considered as the experimental unit, 4 birds per pen).

Table 4. **Egg quality analysis**[1]

Item	Control	Half AD	AD	2-fold AD	3-fold AD	SEM	P-value
Week 2							
Egg weight (g)	61.56	64.01	62.83	63.15	63.55	0.650	0.820
Egg width (cm)	4.37	4.43	4.39	4.40	4.42	0.018	0.847
Egg length (cm)	5.72	5.79	5.80	5.82	5.73	0.023	0.594
Shell strength (kg)	5.56	5.33	4.86	4.97	5.76	1.454	0.239
Shell color	27.38	30.13	28.13	27.25	28.38	0.624	0.625
Albumin height (mm)	8.54	8.90	9.11	9.63	9.90	0.206	0.271
Haugh unit	91.39	93.09	94.39	96.14	97.85	0.947	0.201
Yolk color	4.75	5.00	5.00	5.17	5.38	0.136	0.683
Week 4							
Egg weight (g)	59.21	64.55	62.44	64.00	63.93	1.514	0.417
Egg width (cm)	4.40	4.43	4.39	4.46	4.43	0.013	0.511
Egg length (cm)	5.74	5.81	5.73	5.72	5.86	0.022	0.273
Shell strength (kg)	5.93	5.63	5.74	5.11	5.58	1.267	0.315
Shell color	26.38	26.88	29.57	28.88	27.38	0.584	0.503
Albumin height (mm)	11.21	10.15	9.79	9.91	10.67	0.260	0.387
Haugh unit	103.51	98.48	97.47	97.40	97.37	1.262	0.475
Yolk color	5.57	7.00	5.71	6.38	6.75	0.181	0.620
Week 6							
Egg weight (g)	59.21	64.55	62.44	64.00	63.93	0.812	0.594
Egg width (cm)	3.80	4.40	4.37	3.35	3.80	0.212	0.491
Egg length (cm)	4.91	5.72	5.73	4.26	4.98	0.274	0.409
Shell strength (kg)	5.54	5.67	5.00	5.67	5.20	1.396	0.444
Shell color	28.57	28.75	28.88	27.50	29.00	0.658	0.969
Albumin height (mm)	11.24	10.16	10.26	10.35	10.34	0.349	0.882
Haugh unit	105.77	98.34	98.84	99.37	98.34	1.702	0.615
Yolk color	5.38	5.50	5.75	5.83	4.86	0.270	0.839
Week 8							
Egg weight (g)	61.30	64.66	61.66	61.52	62.35	0.677	0.490
Egg width (cm)	4.34	4.45	4.39	4.37	4.40	0.018	0.379
Egg length (cm)	5.70	5.76	5.69	5.75	5.69	0.029	0.890
Shell strength (kg)	5.65	5.34	4.68	5.19	5.87	1.794	0.295
Shell color	26.86	32.63	28.71	30.71	28.86	0.875	0.271
Albumin height (mm)	9.77	11.09	9.07	9.91	11.03	0.376	0.380
Haugh unit	97.93	101.81	90.26	94.63	102.96	2.545	0.521
Yolk color	5.71	5.38	4.86	5.00	6.00	0.196	0.341

[1] $n=8$ per treatment (one egg from each treatment).
[a–c] Means with different superscripts in the same row differ ($P<0.05$).

Blood Metabolites

The analyzed blood parameters are presented in Table 7. Laying hens fed the half AD diet had lower blood GPT levels compared to that of hens fed the 3-fold AD diet at week 4 ($P=0.010$). Hens fed the control diet had lower blood GOT levels compared to that of hens fed the 2- and 3-fold AD diets on week 4 ($P=0.028$). Consequently, blood GOT levels were higher in hens fed the AD, 2- and 3-fold AD diets compared to that of the hens on the control diet on week 8 ($P<0.001$). Blood UN levels were higher in hens fed the 2-fold diet compared to those fed control and AD diets on week 4 ($P=0.004$); however, UN levels did not differ among the treatments at week 8 ($P>0.05$).

Bioaccumulation of Heavy Metals

Heavy metal concentrations in blood, liver, egg, and feathers are presented in Table 8. Laying hens fed the 2- and 3-fold AD diets had higher blood lead levels compared to that of those fed the control or half AD diet at week 4 ($P=0.004$). Hens fed the AD, 2-, and 3-fold AD diets had higher blood lead levels compared to that of hens fed the control diet on week 8 ($P<0.001$). Hens fed the control diet had lower blood mercury level compared to hens exposed to any level of dietary heavy metals ($P=0.020$).

Discussion

Lead and mercury toxicity is harmful to chickens with the symptoms including depressed growth and development of

Table 5. **Organ parameters**[1]

Item	Control	Half AD	AD	2-fold AD	3-fold AD	SEM	P-value
Week 4							
Absolute weight (g)							
Liver	39.91	41.69	44.23	42.01	44.56	0.776	0.126
Spleen	1.83	1.79	1.98	2.20	2.09	0.077	0.414
Ovary	47.49	46.39	48.40	43.96	45.01	2.823	0.826
Oviduct	62.41	52.19	61.88	56.54	55.68	1.433	0.111
Relative weight (%)							
Liver	2.00^{a}	2.14^{ab}	2.24^{ab}	2.30^{b}	2.31^{b}	0.035	0.024
Spleen	0.09	0.08	0.10	0.12	0.11	0.005	0.081
Ovary	2.44	2.39	2.46	2.39	2.32	0.059	0.957
Oviduct	3.22	2.67	3.13	3.12	2.92	0.083	0.241
Week 8							
Absolute weight (g)							
Liver	39.18^{a}	43.86^{bc}	42.16^{ab}	42.69^{abc}	46.25^{d}	0.535	$<$0.001
Spleen	2.21	2.06	1.90	2.25	1.94	0.061	0.268
Ovary	47.88	49.69	43.30	52.01	47.76	1.240	0.258
Oviduct	67.75	59.88	57.13	58.25	59.00	1.486	0.162
Relative weight (%)							
Liver	2.00^{a}	2.20^{b}	2.14^{ab}	2.12^{ab}	2.20^{b}	0.230	0.049
Spleen	0.11	0.10	0.10	0.11	0.09	0.003	0.125
Ovary	2.44	2.49	2.21	2.57	2.28	0.056	0.223
Oviduct	3.46	3.02	2.93	2.89	2.80	0.080	0.075

[1] $n=8$ per treatment (one selected bird from each treatment).
[a–c] Means with different superscripts in the same row differ ($P<0.05$).

Table 6. **Number of ovarian follicles and weight of F1 follicle**[1]

Item	Control	Half AD	AD	2-fold AD	3-fold AD	SEM	P-value
Week 4							
LYF	4.5	4.3	4.5	4.5	4.3	0.12	0.921
SYF	2.3	2.8	1.8	1.9	2.0	0.16	0.285
LWF	16.6	16.8	15.0	16.9	15.9	0.99	0.976
F1 weight (g)	14.8^{b}	14.6^{b}	14.1^{ab}	13.5^{ab}	12.1^{a}	0.29	0.011
Week 8							
LYF	5.3	5.1	5.1	5.8	5.5	0.14	0.553
SYF	3.0	4.3	4.3	4.1	4.8	0.33	0.549
LWF	11.6	12.5	17.5	16.4	16.6	0.92	0.142
F1 weight (g)	14.3	13.7	13.7	14.7	14.2	0.21	0.593

[1] $n=8$ per treatment (one selected bird from each treatment).
[a–b] Means with different superscripts in the same row differ ($P<0.05$).

anemia, with younger chickens being more susceptible to this toxicity than adults (Salisbury *et al*., 1958; Fimreite, 1970; Simpson *et al*., 1970). Our results showed no differences in egg production, despite laying hens being fed heavy metals up to 3-fold higher than feed formulation registered in South Korea. Meanwhile, birds did not produce inferior eggs and no statistical difference was observed in the measured egg quality parameters among the treatments in the current study. Our results are in accordance with Dauwe *et al*. (2004) who found no difference in egg size or eggshell thickness of the blue tit, *Parus caeruleus*, across a heavy metal pollution gradient. However, thinner and smaller eggs were observed in flycatchers exposed to environmental heavy metal pollution (Eeva and Lehikoinen, 1995). We conducted analyses of the lead and mercury concentrations in the eggs but there were no detected levels in eggs from any of the treatments. Presumably, the heavy metal levels we tested in this study were comparatively lower than would affect growth, egg production rate, and external or internal egg quality.

Exposure to heavy metal can damage organs and tissues from the surface to molecular levels. Once mercury is absorbed, it distributes primarily in the kidney and then the liver of adult birds (Scheuhammer, 1987). In the present study, liver weight relative to body weight was significantly

Table 7. **Blood parameters**[1]

Item	Control	Half AD	AD	2-fold AD	3-fold AD	SEM	P-value
Week 4							
Albumin (g/dL)	1.44	1.49	1.43	1.61	1.60	0.261	0.061
GPT (U/L)	1.58^{ab}	1.56^{ab}	1.40^{a}	1.73^{ab}	2.09^{b}	0.067	0.010
GOT (U/L)	161.09^{a}	164.82^{ab}	173.61^{ab}	181.80^{b}	179.83^{b}	2.358	0.028
UN (mg/dL)	0.69^{a}	0.75^{ab}	0.74^{a}	0.89^{b}	0.80^{ab}	0.018	0.004
GGT (U/L)	27.03	30.00	26.44	27.44	29.09	2.592	0.772
Total protein (g/dL)	5.97	5.81	5.69	6.74	5.91	0.161	0.263
Globulin (g/dL)	4.51	4.32	4.26	5.16	4.35	0.148	0.629
Week 8							
Albumin (g/dL)	1.40	1.53	1.53	1.47	1.47	0.026	0.539
GPT (U/L)	0.64	0.63	0.64	0.98	1.06	0.058	0.568
GOT (U/L)	159.08^{a}	164.59^{ab}	165.38^{b}	174.96^{b}	188.71^{b}	2.540	$<$0.001
UN (mg/dL)	0.85	0.94	0.99	0.84	0.93	0.468	0.826
GGT (U/L)	21.58^{a}	23.60^{b}	22.95^{ab}	27.67^{ab}	27.28^{b}	0.710	0.031
Total protein (g/dL)	5.66	6.00	5.92	6.09	6.24	0.077	0.167
Globulin (g/dL)	4.26	4.48	4.39	4.77	4.63	0.089	0.415

[1] $n=8$ per treatment (one selected bird from each treatment).
[a–c] Means with different superscripts in the same row differ ($P<0.05$).

Table 8. **Concentration of Pb and Hg on blood, liver, egg and feather**[1]

Item		Control	Half AD	AD	2-fold AD	3-fold AD	SEM	P-value
Week 4								
Blood	Hg	27.4	28.4	26.4	26.4	26.9	0.66	0.892
(ppb)	Pb	43.8^{a}	48.9^{ab}	51.5^{abc}	63.9^{c}	58.3^{bc}	2.07	0.004
Liver	Hg	ND	ND	ND	ND	ND	—	—
(ppm)	Pb	ND	ND	ND	ND	ND	—	—
Egg	Hg	ND	ND	ND	ND	ND	—	—
(ppm)	Pb	ND	ND	ND	ND	ND	—	—
Feather	Hg	ND	ND	ND	ND	ND	—	—
(ppm)	Pb	ND	ND	ND	ND	ND	—	—
Week 8								
Blood	Hg	22.7^{a}	28.9^{b}	29.1^{b}	27.1^{b}	28.3^{b}	0.75	0.020
(ppb)	Pb	66.2^{a}	68.8^{ab}	106.1^{c}	93.3^{bc}	119.7^{c}	5.37	$<$0.001
Liver	Hg	ND	ND	ND	ND	ND	—	—
(ppm)	Pb	ND	ND	ND	ND	ND	—	—
Egg	Hg	ND	ND	ND	ND	ND	—	—
(ppm)	Pb	ND	ND	ND	ND	ND	—	—
Feather	Hg	ND	ND	ND	ND	ND	—	—
(ppm)	Pb	ND	ND	ND	ND	ND	—	—

[1] $n=8$ per treatment (one selected bird from each treatment).
[2] ND; not detected.
[a–c] Means with different superscripts in the same row differ ($P<0.05$).

altered on both week 4 and 8 when laying hens were exposed to dietary heavy metals. Particularly, birds fed a diet with the 3-fold AD levels showed statistically heavier relative liver weight compared to those fed the control diet. Previous reports demonstrated lead exposure could change lipid metabolism in chickens, increasing liver cholesterol levels that cause fatty liver issues (Lawton and Donaldson, 1991; Bruggeman *et al*., 1999; Cave *et al*., 2010). Lipid accumulated in the liver resulted in an imbalance in nutrition and fatty liver syndrome by changing the rate of hepatic lipogenesis in chickens (Lee *et al*., 2010). In this respect, our results indicate that heavier liver weight resulted from the slight alteration of fat content in the liver.

Ovarian morphology, such as number and size of follicles, represents egg productivity (Yu *et al*., 1992). When follicles are uniform in order and adequately formed, laying hens can produce sellable eggs. In the present study, birds were not affected by dietary heavy metals in the number of ovarian

follicles produced. The F1 follicle, the largest yellow follicle, controls the follicular hierarchy and the timing of ovulation by regulating the hormones. Immature or ailing chickens have lower progesterone levels and inadequately developed reproductive organs (Bluhm *et al*., 1983). In the present study, most ovarian morphological parameters were not significantly affected by heavy metals except for F1. Decreased F1 follicle weights were observed in hens fed the 2- and 3-fold AD diets. Among the studies dealing with laying hens exposed to heavy metals, to our knowledge, no studies have investigated the number and size of ovarian follicles. We assumed that decreased F1 weight might prolong the retention of follicle formation within the hierarchy because F1 are destined for the next ovulation, and this may negatively effect egg production rate.

Measuring the levels of blood GOT, GPT, and GGT is a well-established and useful diagnostic procedure for detecting liver dysfunction and hepatocellular damage (Reitman and Frankel, 1957; Lin *et al*., 2010). In the present study, laying hens exposed to dietary heavy metals had increased blood GOT, GPT, and GGT activities at both week 4 and 8. Similar results demonstrated that layers fed diets containing 30 mg/kg of lead showed increased GPT and GOT activities, suggesting their production worked to regenerate the liver damage caused by mild lead poisoning (Yuan *et al*., 2013). Moreover, El-Demerdash (2001) reported that rats had biochemical alterations in GOT and GPT activities (serum and liver) in response to oral doses of 0.5 μmol/mL of mercury. Blood urea nitrogen can be used as an indicator to reflect protein catabolism status in the liver of chickens (Robin *et al*., 1987). In the present study, hens fed the 2- and 3-fold AD diets had higher blood UN levels on week 4. The increased level of blood UN, coupled with the GOT and GPT activities observed, may indicate some disruption and hepatic dysfunction due to the heavy metals.

Feathers have been widely used as a bio-indicator for heavy metal exposure. In the present study, we tested heavy metal excretion through feather formation with laying hens; however, lead and mercury were not detected in any treatments at both week 4 and 8. Veerle *et al*. (2004) demonstrated that heavy metal concentrations in feathers are affected by exogenous contamination rather than endogenous deposition. Similarly, Dmowski (2000) demonstrated that lead concentration in feathers indicated mainly external contamination such as by air pollution. Subsequently, heavy metal concentrations in internal tissue (liver) and final product (egg) were not detected among the treatments on week 4 and 8. Laying eggs can be an excretory pathway for endogenous heavy metals in laying hens. In accordance with our results, Dauwe *et al.* (2005) found that blue tits contaminated by lead, cadmium, and mercury showed no clear pattern in producing contaminated eggs. This is because heavy metals transferred from body to egg have been limited and female birds were not efficiently excreting the heavy metal from their body via egg laying. Moreover, Williams and Ternan (1999) revealed that egg white and yolk were derived from recent uptake rather than from nutrient retention in the body. Our results also showed that hens fed the control diet had blood Hg and Pb on week 4 and 8, and this is due to the fact that all hens were raised in a house without separation to reduce the environmental variation. Although the feeding management was conducted to prevent cross-contamination between the heavy metal treatment and control groups, heavy metals are also dispersed via air, and thus a slight contamination seems to have occurred.

In conclusion, our study revealed that exposure of heavy metals up to 30 ppm of lead and 1.2 ppm of mercury might be less risky for saleable egg production. However, 8-week exposure of dietary heavy metals induced hepatic dysfunction by increasing blood GPT and GOT levels. Therefore, in-feed heavy metals should be eliminated or reduced as much as possible not only to prevent potential risk to future performance for laying hens, also to ensure the food safety for humans.

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