

Excess dietary fluoride affects laying performance, egg quality, tissue retention, serum biochemical indices, and reproductive hormones of laying hens

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ABSTRACT The present study aimed to evaluate the effects of excess dietary fluoride (F) on laying performance, egg quality, tissue retention, serum biochemical indices, and serum reproductive hormones of laying hens. A total of 384 Hy-Line Gray hens, 37 wk old, were treated with sodium fluoride added to a corn-soybean meal basal diet at 0, 400, 800, and 1200 mg fluoride/kg feed. The results showed that dietary F levels at 800 and 1200 mg/kg markedly decreased ADFI, laying rate, average egg weight, and increased feed conversion ratio (FCR) ($P < 0.05$). Dietary F levels at 800 and 1200 mg/kg dramatically decreased the egg quality of albumen height, yolk color, eggshell strength, and eggshell thickness, and on the 49th D, 400 mg/kg F group significantly decreased the eggshell strength, compared to those of control group. Fluoride residues in tissues of hens were increased significantly with the increase of dietary F supplemental levels ($P < 0.05$).

Fluoride concentrations were generally high in feces, eggshell, tibia, kidney, and ovary, and the highest in feces, following with eggshell and tibia, lower in kidney and ovary, and the lowest in serum. Serum uric acid levels and alanine aminotransferase activity increased significantly ($P < 0.05$), and glucose, triglycerides, and phosphorus decreased significantly ($P < 0.05$) in response to dietary F concentration, compared to those of the control group, respectively. Dietary F supplementation at 1200 mg/kg significantly decreased ($P < 0.05$) the estrogen concentrations in serum, compared to those of the control group. Concentrations of progesterone in the fluoride-treated groups were significantly ($P < 0.05$) decreased relative to those of the control group. In conclusion, these results indicated that the excessive ingestion of F has had a detrimental effect on egg laying rate and quality of eggs by damaging the function of the liver, kidney, and ovary of laying hens.

Key words: fluoride, laying hen, laying performance, egg quality, tissue retention

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INTRODUCTION

Fluorine (F) is one of the elements necessary for animal health (Jha et al., 2013); however, chronic exposure to large amounts of F increases body burden (Pollick, 2004). Fluoride is an ionic compound of fluorine and occurs naturally at varying levels in rocks, water, and soil due to its high responsiveness (Choi et al., 2012). Drinking water containing high concentration of fluoride is the primary source of human exposure to the F environment worldwide, particularly in China and India (Zhou et al., 2013a). Accumulated studies have shown that, in addition to hard tissues, the excessive exposure of F causes a diversity of pathological changes in soft tissues, includ-

ing reproductive tissues (Darmani et al., 2001; Wang et al., 2017), liver (Zhan et al., 2006a; Cao et al., 2013), kidney (Zhan et al., 2006b; Singh et al., 2017), thymus (Yin et al., 2016), and brain (Chinoy and Patel, 2000; Vani and Reddy, 2000). Most of these investigations concerning F have been done in rats (Adedara et al., 2017; Campos-Pereira et al., 2017), mice (Darmani et al., 2001; Wang et al., 2017), rabbits (Ma et al., 2012), human beings (Bergandi et al., 2010; Kimsa-Dudek et al., 2018), and fish (Mukhopadhyay et al., 2015; Singh et al., 2017), but rarely in laying hens, which plays an key role in stockbreeding.

Our previous work found that antioxidant ability of laying hens was susceptible to disruption by F at concentrations that are enough to produce other manifestations of toxicity (Miao et al., 2017a). However, the toxic effects of F on laying performance and egg quality of laying hens have rarely been conducted. The purpose of this study was, therefore, to investigate the effects

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Table 1. Ingredient and nutrient composition of the basal diet (air-dry basis).

Ingredients	Composition, %	Nutrient ²	Composition, %
Corn	65.00	ME, Mcal/kg	2.65
Soybean meal (44.20% CP)	20.50	CP, %	15.64
Fish meal	2.50	Ca, %	3.51
Limestone	7	TP, %	0.65
Premix ¹	5.00	Lys, %	0.82
		Met, %	0.36
		Typ, %	0.17
Total	100.00	(Met+Cys), %	0.65

¹The premix provided the following per kg of the diet: vitamin A, 7600 IU; vitamin D3, 2000 IU; vitamin E, 15 IU; vitamin K, 2 mg; thiamine, 1 mg; riboflavin, 8.5 mg; calcium pantothenate, 50 mg; niacin, 32.5 mg; pyridoxine, 8 mg; biotin, 2 mg; folic acid, 5 mg; vitamin B12, 5 mg; choline, 500 mg; Mn, 65 mg; I, 1 mg; Fe, 65 mg; Cu, 10 mg; Zn, 66 mg; and Se, 0.12 mg.

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

Crude protein (CP), calcium (Ca), phosphorus (P), and total phosphorus (TP).

of excess F on laying performance, egg quality and to examine the tissue retention, serum biochemical indices, and serum reproductive hormones of laying hens that had been exposed to sodium fluoride (NaF).

MATERIALS AND METHODS

The experiment was carried out according to the Chinese guidelines for animal welfare and approved by the Animal Welfare Committee of the College of Animal Sciences of the University of Zhejiang (No. ZJU2013105002), Hangzhou, China.

Birds and Housing

A total of 384 Hy-Line Gray hens with similar performance, 37 wk of age, obtained from a commercial layer farm (Hangzhou, China), were randomly distributed to 4 treatments with 6 replicas of 16 hens. Four hens in an individual cage (45 × 45 × 50 cm) equipped with 2 nipple drinkers and 1 feeder were kept in a ventilated room with a temperature between 28°C and 33°C and relative humidity of 65 ± 5%. The hens were kept in 3-layer cages of the full scale and fed ad libitum twice a day at 07:20 am and 15:20 pm and the water were available all the time. The hens were maintained under a controlled environmental condition, and a 16-hr photoperiod of daily light was maintained.

Experimental Diets

All laying hens were fed with corn-soybean meal basal diet (31.19 mg/kg F) for 10 D and then were randomly allocated into 4 groups that were fed a basal diet (control), or a basal diet supplemented with 400, 800, and 1200 mg F/kg feed from NaF (NaF, 99% purity, Hushi, Shanghai, China) for 49 D. The diets were formulated to meet or exceed NRC (1994) recommendations for all nutrients. The composition and nutrient levels of the formulated corn-soybean meal basal diet are listed in Table 1, with the F concentrations of the 4 diets shown in Table 2.

Table 2. Fluoride (F) concentrations of 4 experimental diets (mg F/kg feed).¹

Item	Dietary F supplementation (mg/kg)	Measured F concentrations (mg/kg)
Control group	0	31.19
400 mg/kg group	400	431.38
800 mg/kg group	800	831.70
1200 mg/kg group	1,200	1,237.16

¹Corn-soybean meal basal diet (31 mg/kg F) were supplemented with 0, 400, 800, and 1200 mg F/kg feed from sodium fluoride (NaF, 99% purity, Hushi, Shanghai, China).

Sample Collection

Feed residues were collected and weighed weekly to estimate the average feed intake. Eggs obtained from each replicate were counted and weighted daily to calculate the laying rate, egg weight, and feed conversion ratio (FCR). Health status and mortality were visually observed and recorded daily throughout the experimental period. On the 49th D, 12 layers (2 layers per replicate) were selected in each group and finally sacrificed after the 12-hr fast (water offered ad libitum) to collect the tibia, kidney, and ovary. The samples were rinsed twice with cold iced PBS and dried with filter paper to prevent blood contamination. Blood samples were collected from the jugular vein and centrifuged at 958 × G for 10 min to separate the serum. Also, 30 eggs (5 eggs of each replicate) for each treatment were randomly selected for the quality of eggs on the 21st and 49th D. A total of 15 eggs from each group were collected separately to detect F in albumen (ALB), yolk, and egg shells (eggshell membrane removed) over the 49th D. Hens' feces (12 hens of each treatment) were collected for the detection of F concentration on the 47th D of the test. All samples were stored at -80°C for analysis.

Experimental Parameters Measured

The contents of F in the feces, tibia, kidney, ovary, albumin, yolk, eggshell, and serum were measured

using a potentiometric method utilizing anionic selective electrode (Leici, PF-1 01, Shanghai, China) (Miao et al., 2017b). The eggs were weighed and cracked and then the height of the ALB, the Haugh unit, the yolk color, the thickness of the eggshell and strength were determined with a digital egg tester (DET-6000; Nabel Co. Ltd., Kyoto, Japan). The thickness of the eggshell (without the eggshell membrane) was measured using the middle part of the eggshell. Serum total protein levels (**TP**), of ALB, urea nitrogen (**BUN**), glucose (**GLU**), urea acid (**UA**), calcium (**Ca**), phosphorus (**P**), aspartate aminotransferase (**AST**), and alanine aminotransferase (**ALT**) activity were analyzed and calculated by the protocols of commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

The levels of progesterone (**P4**) and estradiol (**E2**) in the serum were measured by Radioimmunoassay (**RIA**) Assay kits (IBL International, Munich, Germany) according to the manufacturer's instructions.

Statistical Analysis

Data were statistically analyzed by one-way ANOVA of SPSS 20.0 for Windows (SPSS Inc., Chicago, IL). If significant differences were found ($P < 0.05$), Tukey post hoc tests would be performed.

RESULTS

Laying Performance

As shown in Table 3, dietary F supplementation at 800 and 1000 mg/kg significantly decreased the laying rate, egg weight, ADFI, and FCR. Compared with the control group, laying rate in 800 and 1000 mg/kg F groups was decreased by 33.49% ($P < 0.05$) and 57.95% ($P < 0.05$), ADFI in 800 mg/kg and 1000 mg/kg F groups was decreased by 13.75% ($P < 0.05$) and 28.79% ($P < 0.05$), average egg weight was significantly decreased by 5.70% ($P < 0.05$) and 6.19% ($P < 0.05$), FCR was significantly increased by 31.53% ($P < 0.05$) and 76.85% ($P < 0.05$), respectively.

Table 3. Effects of dietary fluoride (F) levels on laying performance.¹

Laying performance	F concentration (mg/kg)				SEM	P-value
	Control	400	800	1200		
Laying rate (%)	89.75 ^a	87.21 ^a	59.69 ^b	37.74 ^c	2.196	0.000
Egg weight (g)	57.37 ^a	56.52 ^a	54.10 ^b	53.85 ^b	0.492	0.000
ADFI (g)	106.83 ^a	103.13 ^a	92.16 ^b	76.07 ^c	1.887	0.000
FCR	2.03 ^a	2.15 ^a	2.67 ^b	3.59 ^c	0.081	0.000
Mortality	0.347 ^b	0.347 ^b	0.868 ^{a,b}	1.910 ^a	0.388	0.002

¹Results are the mean \pm SEM of 6 replicates, with 16 hens per replicate. ADFI = average daily feed intake; FCR = feed conversion ratio.

^{a-c}Means within a row with different superscript differ significantly ($P < 0.05$).

Egg Quality

The data of ALB height, yolk color, Haugh unit, eggshell strength, and eggshell thickness are presented in Table 4. Adding F to basal diet significantly decreased ($P < 0.05$) the levels of eggshell strength and eggshell thickness, both on the 21st and 49th D, compared to those of the control group, respectively. Moreover, the levels of eggshell strength and eggshell thickness on the 49th D are lower than those on the 21st D. Dietary F supplementation at 800 and 1000 mg/kg significantly decreased the ALB height ($P < 0.05$) on the 21st D and yolk color ($P < 0.05$) on the 49th D, compared to those of the control group, respectively.

Effects of Dietary F Levels on F Contents in Partial Organs and Eggs of Laying Hens

Table 5 showed that the residues of F in feces, eggshell, tibia, kidney, ovary, yolk, ALB, and serum of laying hens increased significantly with the increase of dietary F levels ($P < 0.05$). Fluoride concentrations were generally high in feces, eggshell, tibia, kidney and ovary, and the highest in feces, following with eggshell and tibia, lower in kidney and ovary, and the lowest in serum.

Effects of Dietary F Levels on Biochemical Indices

As shown in Table 6, serum UA levels and ALT activity increased significantly ($P < 0.05$), but AST/ALT ratio, GLU, TG, and P levels decreased significantly ($P < 0.05$) in response to dietary F concentration, compared to the control groups, respectively.

Effects of Dietary F Levels on Serum E2 and P4 Levels

Concentrations of E2 and P4 in the serum of laying hens were presented in Figure 1. Dietary F supplementation at 1200 mg/kg significantly decreased ($P < 0.05$) the E2 concentrations in serum, compared to the control group. Progesterone levels in the NaF-treated groups were markedly ($P < 0.05$) down-regulated compared to those in the control group.

DISCUSSION

This study was conducted to assess excess dietary F levels on laying performance, egg quality, tissue retention, serum biochemical indices, and reproductive hormones of laying hens. It is well known that high F concentration is harmful to human and animal and its accumulation leads to a large number of hematological, hepatic, renal, cerebral, cardiovascular, and neurological disorder (Chinoy and Patel, 2000; Vani and Reddy, 2000; Zhan et al., 2006a; Zhan et al., 2006b; Cao

Table 4. Effects of dietary fluoride (F) levels on egg quality.¹

Item	Trail period (day)	F concentration (mg/kg)				SEM	P-value
		Control	400	800	1200		
Albumen height (mm)	21st	7.45 ^a	7.23 ^{a,b}	7.01 ^{b,c}	6.73 ^c	0.120	0.000
	49th	6.57	6.44	6.44	6.37	0.090	0.212
Yolk color	21st	5.78	5.73	5.55	5.45	0.152	0.138
	49th	5.78 ^a	5.56 ^a	4.84 ^b	5.07 ^b	0.081	0.000
Haugh unit	21st	86.09	84.23	83.58	83.03	1.138	0.078
	49th	82.98	82.75	80.43	81.81	1.102	0.126
Eggshell strength (kgf/m ²)	21st	4.07 ^a	4.01 ^a	3.28 ^b	2.42 ^c	0.075	0.000
	49th	4.23 ^a	3.39 ^b	2.88 ^c	1.77 ^d	0.174	0.000
Eggshell thickness (mm)	21st	0.337 ^a	0.343 ^a	0.340 ^a	0.310 ^b	0.009	0.008
	49th	0.382 ^a	0.367 ^a	0.345 ^b	0.313 ^c	0.006	0.000

¹Results are the mean and SEM of 6 replicates, with 5 eggs per replicate.

^{a-d}Means within a row with no common superscripts differ significantly ($P < 0.05$).

Table 5. Effects of dietary fluoride (F) levels on concentration of F in tissues of laying hens.¹

Item	F concentration (mg/kg)				SEM	P-value
	Control	400	800	1200		
Feces	49.81 ^d	174.94 ^c	345.25 ^b	562.15 ^a	17.45	0.000
Eggshell	3.94 ^d	52.46 ^c	162.27 ^b	204.91 ^a	2.21	0.000
Tibia	36.19 ^d	82.77 ^c	121.25 ^b	138.55 ^a	2.88	0.000
Kidney	1.06 ^d	6.06 ^c	9.27 ^b	10.07 ^a	0.12	0.000
Ovary	0.67 ^d	4.92 ^c	6.85 ^b	9.06 ^a	0.09	0.000
Yolk	0.69 ^c	2.47 ^b	2.60 ^a	2.71 ^a	0.04	0.000
Albumin	0.39 ^d	1.42 ^c	1.68 ^b	1.93 ^a	0.07	0.000
Serum	0.007 ^d	0.058 ^c	0.109 ^b	0.141 ^a	0.004	0.000

¹Results are the mean and SEM of 6 replicates.

^{a-d}Means within a row with no common superscripts differ significantly ($P < 0.05$).

Table 6. Effects of dietary fluoride (F) levels on serum biochemical indices of laying hens.¹

Item	F concentration (mg/kg)				SEM	P-value
	Control	400	800	1200		
AST(IU/L)	115.48	93.96	105.86	101.34	8.34	0.126
ALT(IU/L)	18.56 ^b	30.31 ^{a,b}	28.11 ^{a,b}	38.45 ^a	4.97	0.009
AST/ALT	4.33 ^a	2.94 ^{a,b}	3.15 ^{a,b}	2.47 ^b	0.592	0.047
BUN (mmol/L)	3.11	3.22	2.74	2.97	0.30	0.445
ALB (g/L)	22.58	24.15	23.77	23.42	1.38	0.711
TP (mg/ml)	51.51	53.44	54.70	53.48	4.64	0.921
UA (mg/L)	38.94 ^b	43.66 ^{a,b}	46.29 ^{a,b}	50.04 ^a	3.15	0.020
GLU (mmol/L)	14.17 ^a	12.55 ^a	7.78 ^b	7.26 ^b	0.83	0.000
TG (mmol/L)	27.55 ^a	20.10 ^{a,b}	7.69 ^b	7.65 ^b	4.96	0.005
Ca (mmol/L)	5.08	5.43	4.76	4.68	0.617	0.618
P (mmol/L)	1.73 ^a	1.47 ^{a,b}	1.13 ^b	1.29 ^b	0.135	0.005

¹Results are the mean and SEM of 6 replicates, with 12 hens per replicate.

²Total protein (TP), albumen (ALB), urea nitrogen (BUN), urea acid (UA), calcium (Ca), phosphorus (P), aspartate aminotransferase (AST) and alanine aminotransferase (ALT), triglyceride (TG), and glucose (GLU).

^{a,b}Means within a row with no common superscripts differ significantly ($P < 0.05$).

et al., 2013; Yin et al., 2016; Singh et al., 2017). It has been demonstrated that more than 700 mg/kg dietary F supplementation can reduce hens' laying performance (Hahn and Guenter, 1986a). In the present study, we found that 400 mg/kg dietary F supplementation did

not change the laying rate, ADFI, FCR, and average egg weight. However, when dietary F supplementation up to 800 or 1200 mg/kg would markedly decrease laying rate, ADFI, and average egg weight; increase FCR of laying hens, which agreed with our previous observations (Miao et al., 2017a).

Similarly, Hahn and Guenter (1986a) found that dietary F concentration up to 1000 mg/kg significantly depressed feed intake and laying rate. Zhou et al. (2013a) found that the total number of each type of follicle was changed in the sodium fluoride-treated groups: the number of small follicles increased, and the large follicle number decreased. It may be one mechanism of decrease in laying rate of hens exposed to F.

When taking out the feeding experiment, we observed that eggshell became thinner and crisper in 1200 mg/kg F group. In the following egg quality detection, we found that dietary F levels at 800 or 1200 mg/kg numerically decreased album height on the 21st D, which agreed with our previous observations (Miao et al., 2017b), but the change disappears on the 49th D. Now we cannot explain the observation. On the 49th D, excess dietary F levels decreased the yolk color. The reason for this observation may be the lower ADFI in high F group. As we all know that coloration of the yolk depends on the accumulation of carotenoids, which are synthesized de novo only by higher plants, algae, bacteria, and fungi (Goodwin, 1980). However, the laying hens are unable to synthesize the xanthophylls, maintaining a uniform and coherent color depends directly on the quantity, the coloring capacity and the stability of the dietary carotenoids (Nys, 2000). If the ADFI were decreased, the yolk color would reduce. The eggshell is the natural packing material for the egg contents, and as a result, it is crucial to gain high shell strength and thickness, to resist all impacts on eggs during the production chain (Bain, 1990). The concept of shell quality is frequently used as a synonym for eggshell strength and thickness and indicates the ability of egg shells to withstand externally applied forces without cracking or rupture (Ketelaere et al., 2002). Broken eggs cause

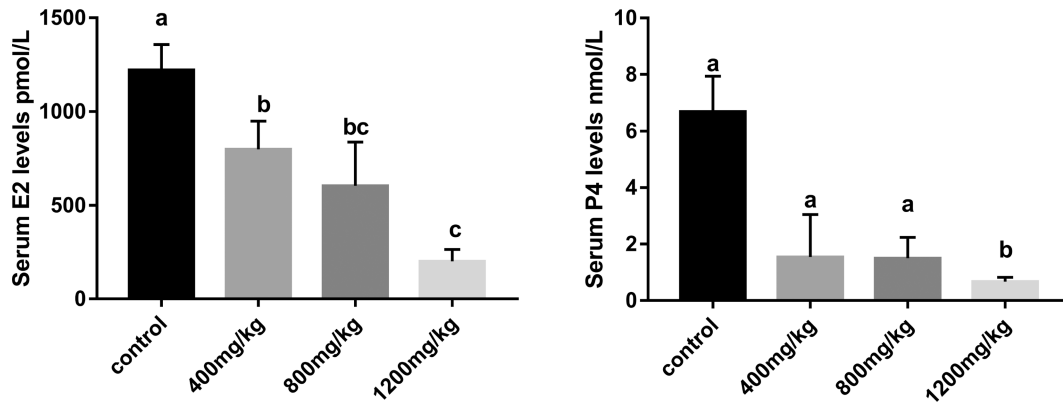


Figure 1. Effects of dietary fluoride (F) levels on serum estrogen (E2) and progesterone (P4) levels of laying hens. Values are expressed as the means \pm SEM ($n = 6$). Different lowercase letters indicate significant differences ($P < 0.05$).

economic damage, including that they cannot be sold as premium eggs, and the appearance of capillary cracks strengthens the risk of bacterial contamination and food safety (Mertens et al., 2006). Therefore, eggshell strength and eggshell thickness are critical indicators of eggs. The effects of F were found to observably decrease eggshell strength and eggshell thickness in the present study, which was consistent with our team's previous research (Miao et al., 2017b).

The reproductive system of female animals is susceptible to disorders following exposure to F (Zhou et al., 2013a; Fu et al., 2014). Exposure to F could be deposited in the ovary and affect the architectures and components of the ovary, and also caused abnormalities in ovulation, such as the promotion of follicular atresia and hormonal irregularity (Zhou et al., 2013b; Miao et al., 2017b). In the present study, we found that the F concentrations in ovary were increased response to dietary F supplementation, which agreed with previous observations of Zhou et al. (2013b). It was speculated that much F concentration in ovary destroyed its structure and resulted in dysfunction. It is confirmed that the steroid hormones E2 and P4 play a key role in the growth and differentiation of reproductive organs (Da Silva Faria et al., 2010). Estrogen modulates steroidogenesis, promotes proliferation of ovary granulosa cell, and maintains the general development of ovary follicles (Drummond and Findlay, 1999). In the present study, we observed a markedly decrease in serum levels of E2 and P4, which agreed with the results of Zhou et al. (2013b), which found that F exposure lead to significant decreases in E2 and P4 levels in the rat's serum; and the endometrial cells became larger, and the endometrial glands became hypertrophic.

The present study showed that F concentrations were generally high in feces, eggshell, tibia, kidney, and ovary, and the highest in feces, following with eggshell and tibia, lower in kidney and ovary, and the lowest in serum, which was agreed with previous observations (Hahn and Guenter, 1986b; Miao et al., 2017b). The present results suggested F levels in ALB and yolk were

much lower than those in eggshell, which agreed with previous studies reporting that F was mainly deposited in eggshell (Guenter and Hahn, 1979; Górecki et al., 2006; Miao et al., 2017b).

Fluorine is a pro-bone element and is mainly distributed in hard tissues, like bones and teeth (Song et al., 2011). In the layers, P is necessary for the replacement of tissue metabolites such as nucleotides and phospholipids, to maintain skeletal integrity, and for egg production. Calcium is the primary structural element in the eggshell, and large amounts of Ca are needed to synthesize the shell (Kebreab et al., 2009). In this study, serum P concentrations decreased, while serum Ca concentrations did not change with dietary F supplementation, which destroyed the P-Ca equilibrium. It might be a mechanism of reducing the eggshell strength and eggshell thickness. In the advanced study, severe hypoglycemia was associated with increased risk of death, cancer, and conditions affecting the respiratory and the digestive system (Zoungas et al., 2010). In our study, we also found that serum GLU levels decreased with dietary F concentrations.

The liver plays an essential role in lipid metabolism, several stages of lipid synthesis and transport (Ghadir et al., 2010). The previous study found that serum TG concentrations were significantly decreased in patients with hepatic disease (Ghadir et al., 2010). In this study, we also indicate that serum TG concentrations decreased with dietary F levels. Aminotransferases serum assays are the most common laboratory tests for the detection of hepatic diseases and indicate that serum aminotransferase concentration is associated with mortality caused by hepatic disease, individuals with a slight increase in the concentration of aminotransferases, higher mortality (Hyeon et al., 2004). Alanine aminotransferase is the more specific marker of hepatocellular lesions (Kew, 2000), AST/ALT ratios decreased, indicating hepatic lesions (Cohen and Kaplan, 1979). In our results, we found that serum ALT levels increased, and AST/ALT ration decreased in high F-groups. It has been conjectured

that the high levels of F food have destroyed the function of the liver.

Uric acid is a product of purine derivatives. After being filtered, UA is both reabsorbed and excreted in the proximal tubule by a voltage-sensitive urate channel and a urate-anion exchange mechanism (Siu et al., 2006). In rats with renal disease, there is a decrease in urinary excretion of the UA and an increase in plasma UA (Vaziri et al., 1995). In the current study, we found that the level of serum UA markedly increased, which indicated that the kidney of hens exposed to F was damaged.

In conclusion, these results showed that the dietary supplementation of 400 mg/kg F to Hy-Line Gray Hens did not alter the laying rate, egg quality, ADFI, and FCR. However, when dietary supplementation F is up to 800 and 1200 mg/kg, it would have significantly adverse effects on laying performance. Tissue F concentrations, serum biochemical indices, serum reproductive hormones indicate that the excessive ingestion of F damages the liver, kidney, and ovary of laying hens, and ultimately decreases the rate of laying and quality of the eggs.

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