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Evaluation of δ-Aminolevulinic Acid on Serum Iron Status, Blood Characteristics, Egg Performance and Quality in Laying Hens

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ABSTRACT : Effects of dietary δ-aminolevulinic acid (ALA) supplementation on serum iron status, blood characteristics, egg production and quality were examined in laying hens in an 8-week feeding trail. Two hundred and forty (Hy-line brown, 40-week-old) layers were randomly assigned to four dietary treatments with ten replications (six layers in adjacent three cages). Dietary treatments included: 1) CON (basal diet), 2) ALA1 (CON+ALA 5 ppm), 3) ALA2 (CON+ALA 10 ppm) and 4) ALA3 (CON+ALA 15 ppm). All nutrient levels of diets were formulated to meet or exceed NRC (1994) recommendations for laying hens. During the entire experimental period, differences of serum iron concentration and total iron binding capacity (TIBC) were significantly increased in ALA1 supplemented treatment (quadratic effect, $p<0.05$). The difference of total protein between 8 and 0 weeks was significantly higher in ALA2 treatment than CON treatment (quadratic effect, $p<0.05$). No significant effects were observed on hemoglobin, WBC, RBC, lymphocyte and albumin concentrations. Egg production and egg weight were not influenced by the ALA supplementation. Egg yolk index was also significantly higher in ALA3 treatment than CON treatment at the end of 4 and 8 weeks (linear effect, $p<0.05$). Haugh unit was increased in ALA3 treatment compared to CON and ALA1 treatments at the end of 8 weeks (linear effect, $p<0.05$). However, egg shell thickness, breaking strength and yolk color unit were not affected by the ALA supplementation. In conclusion, dietary ALA supplementation at a level of 5 ppm can affect iron concentration in serum while higher levels (10 or 15 ppm) have some beneficial influences on blood profiles and egg quality. (**Key Words :** δ-Aminolevulinic Acid, Iron Status, Egg Quality, Laying Hens)

INTRODUCTION

It is well accepted that iron plays an important role in nutrition of both human and animals. Iron acts as a component of hemoglobin in red blood cell, as myoglobin in muscle, as transferrin in serum, as uteroferrin in placenta, as lactoferrin in milk and as ferritin and hemosiderin in liver (Ducsay et al., 1984). In addition, iron is also necessary for several enzymatic processes and metabolism steps in the body. According to NRC (1994), the requirement of iron for poultry ranges from 50 to 120 mg/kg of diet. However, most of above mentioned functions can't be accomplished when iron present as simple ion form, while over 90% of the iron is present in complex bound to porphyrins in some different ways. Therefore, iron supplementation in mineral premix (generally as ferrous sulfate form), as well as additional organic form iron, has been widely applied when formulate hens diet due to the low availability. Such suggestion was approved by Park et al. (2004), who reported that both inorganic and organic form of iron supplementation over the requirement had positive effects on egg quality parameters, with the organic form presented higher efficiency than inorganic form.

The major functional form of iron is heme, which is the prosthetic group of hemoglobin, myoglobin and the cytochromes. Biosynthesis of heme starts in the mitochondrial matrix by condensing succinyl-CoA with glycine to form δ-aminolevulinic acid (ALA). Then ALA dehydrates condenses 2 ALAs to porphobilinogen, which is subsequently converted to protoporphyrin IX. Ferrochelatase (an iron-sulfur enzyme) catalyzes the last step in heme biosynthesis by inserting iron into protoporphyrin IX to produce heme (Ponka, 1999; Atamna, 2004). Therefore, it can be hypothesized that extra ALA addition may stimulate the reactions to produce more heme. During this process, simple ionic iron can be converted to heme iron, which has higher efficiency and utility for animals.

Generally, ALA is only used for photodynamic therapy

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Table 1. Basal diet composition (as-fed basis)

Ingredients	%
Corn	50.4
Soybean meal (CP 46%)	18.7
Wheat grain	10.0
Corn gluten meal	2.00
Wheat bran	5.00
Animal fat	4.40
Limestone	7.50
Tricalcium phosphate (P 18%)	1.40
Salt	0.30
DL-methionine (50%)	0.10
Vitamin premix[1]	0.10
Mineral premix[2]	0.10
Total	100
Chemical composition[3,4]	
ME (Kcal/kg)	2,904
Crude protein (%)	15.5
Lysine (%)	1.80
Methionine (%)	0.32
Calcium (%)	3.25
Phosphorus (%)	0.61

[1] Provided per kg of diet: 125,000 IU vitamin A; 2,500 IU vitamin D_3; 10 mg vitamin E; 2 mg vitamin K_3; 1 mg vitamin B_1; 5 mg vitamin B_2; 1 mg vitamin B_6; 15 mg vitamin B_{12}; 500 mg folic acid; 35,000 mg niacin; 10,000 mg Ca-Pantothenate and 50 mg biotin.

[2] Provided per kg of diet: 8 mg Mn; 60 mg Zn; 25 mg Cu; 40 mg Fe; 0.3 mg Co; 1.5 mg I and 0.15 mg Se.

[3] Calculated values.

[4] The total concentrations of Zn, Fe, Cu in diet were 88, 94 and 32 mg/kg, respectively.

of tumor due to its expensive price. Recently, a novel method with low cost for producing ALA by bacteria fermentation has been developed, therefore, it provides the possibility to use ALA in livestock feed industry. Previous study which conducted by Min et al. (2004) suggested that dietary ALA supplementation in weaned pig's diet had beneficial effects on growth performance and blood parameters. In addition, Chen et al. (2008) also observed dietary ALA administration can improve nutrients digestibility and immune response in weanling pigs. However, few studies have been performed in poultry until now. The objective of current study was to evaluate whether feeding ALA supplemented diet for laying hens would positively influence iron turnover and utilization, subsequently improve the performance and egg quality of laying hens.

MATERIALS AND METHODS

Experimental design, animals and diets

Two hundred forty 40-week-old ISA brown commercial laying hens were selected for this 8-weeks feeding trial. Hens were allocated into four dietary treatments with ten replications per treatment according to a completely randomized block design. Each replication of six hens was assigned in three adjacent cages providing two hens per cage. All the cages were equipped with nipple drinkers and common trough feeders. Therefore, each replication represented three cages in which the hens were fed from the same feed trough. Dietary treatments were as follow: 1) CON (basal diet); 2) ALA1 (basal diet+ALA 5 ppm); 3) ALA2 (basal diet+ALA 10 ppm) and 4) ALA3 (basal diet+ ALA 15 ppm). The ALA (EASY BIO System, Inc., Korea) was produced by recombinant *Escherichia coli* containing the *Rhodobacter capsulatus* hemA gene. All nutrient levels of diets were formulated to meet or exceed the NRC (1994) requirements of laying hens and provided by mash form. Before the start of experiment, hens were provided with basal diet for a 7 d adjustment period. The composition of the experimental diet is shown in Table 1. Hens were housed per cage in a three-tier cage system. An environmentally controlled room was provided at 21°C by sensor monitored the inside temperatures and adjusted ventilation fans to control the temperature. Hens were maintained on a 17 h:7 h light:darkness photoperiod following light stimulation. Feed and water were provided *ad libitum*.

Sampling and measurements

At the initial of experiment, 10 laying hens were randomly selected from each treatment (one hen in each replication) and blood samples were collected from wing vein using sterilized injector. Then, samples were transfer into either vacuum or K_3EDTA vacuum tube (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA). Same laying hens were also collected blood samples at the end of experiment. For serum analysis, blood samples were centrifuged at 2,000×g at 4°C for 20 min and serum was separated. Total protein, albumin, hemoglobin, iron and total iron binding capacity (TIBC) concentrations in serum were analyzed by automatic biochemistry blood analyzer (HITACHI 747, Japan). Whole blood samples were analyzed for WBC, RBC and lymphocyte concentrations by the automatic blood analyzer (ADVIA 120, Bayer, Tarrytown, NY, USA).

Eggs were collected and counted daily throughout the experimental period. The egg production was calculated using total egg numbers divided by numbers of hens per cage. Egg weight was measured by electronic scale. At the initial and every four weeks interval, thirty eggs, except soft and broken eggs, from each treatment were selected for analyzing egg quality. Eggshell breaking strength was measured by using an egg shell strength meter (Ozaki MFG. Co., Ltd., Japan). Egg shell thickness was measured at three locations (all cell, equator and sharp end) of eggs by dial pipe gauge (Ozaki MFG. Co., Ltd., Japan). Egg yolk color was evaluated by yolk color fan (Roche, Switzerland). Egg

Table 2. Effect of ALA supplementation on serum iron status in laying hens[1]

Items	CON	ALA1	ALA2	ALA3	SEM[2]
Hemoglobin (g/dl)					
0 week	8.90	8.60	9.18	7.25	0.58
8 weeks	10.1	10.9	11.2	9.7	1.03
Difference (0 week-8 weeks)	1.18	2.33	2.00	2.45	2.07
Iron (μg/dl)					
0 week	803	719	817	760	41
8 weeks	775	827	833	818	21
Difference (0 week-8 weeks)[3]	-28.1	108	15.8	58.4	28.2
TIBC (μg/dl)					
0 week	827	773	866	806	28
8 weeks	875	907	912	873	32
Difference (0 week-8 weeks)[3]	48.1	135	45.2	66.9	24.6

[1] Each mean represents ten observations per treatment. CON, basal diet; ALA1, basal diet with ALA 5 ppm; ALA2, basal diet with ALA 10 ppm; ALA3, basal diet with ALA 15 ppm; TIBC, total iron binding capacity.
[2] Pooled standard error of the means. [3] Quadratic effect, $p<0.05$.

yolk index was calculated according to the method of Funk (1948) based on the height and diameter of yolk. Haugh unit were measured according to the HU formula (Eisen et al., 1962) based on the height of albumen as determined using a micrometer.

Statistical analysis

Statistical analysis was performed by using General Linear Models procedure in a completely randomized block design with the SAS software program (SAS Institute, 1996). Significant differences among treatment means were determined at $p<0.05$ by least significant different test. In addition, orthogonal comparisons were conducted by polynomial regression to measure the linear and quadratic effects for increasing dietary concentrations of supplemental ALA.

RESULTS AND DISCUSSION

Effects of ALA supplementation on serum iron status are shown in Table 2. The serum hemoglobin concentration was not affected by ALA supplementation throughout the experiment. There were no significant differences of iron and TIBC concentrations at the beginning and ending of experiment. However, the difference of serum iron concentration was greater in ALA1 treatment than other treatments (quadratic effect, $p<0.05$). Similarly, difference of serum TIBC was significantly higher in ALA1 treatment than other dietary treatments (quadratic effect, $p<0.05$).

The differences of serum iron and TIBC concentrations between initial basic level and ending level presented significant effects, which the 5 ppm ALA supplemented treatment shown greater improvement. This result indicated that iron status in blood can be improved by ALA supplementation. However, iron concentration and TIBC did not present significant differences when they were determined at the end of experiment. This may due to the numerical variation at the beginning of experiment (ranged from 719 to 817 and 773 to 866 μg/dl, respectively). Similar with current result, Min et al. (2004) also observed dietary 0.2% ALA supplementation for weaned pigs can increase serum iron and TIBC concentration. In addition, they also observed increased RBC and hemoglobin concentrations by 0.2% ALA supplementation. In another nursery pig experiment, Mateo et al. (2006) reported 0.05% ALA addition increased RBC concentration as well, but decreased hemoglobin concentration. Differently, in our study, both RBC and hemoglobin concentrations were not affected by ALA addition. These inconsistent results form different experiments may because of different experimental animals and addition dosages. As far as we know, this research is the first report of dietary ALA administration in laying hens, therefore, no other similar laying hens experiment is available to compare with current study. It should be note that the greater effect of iron was observed in 5 ppm ALA added treatment other than higher dosage treatment (10 ppm or 15 ppm). Interestingly, according to a recent study, Chen et al. (2008) also observed 10 ppm ALA supplementation in piglet diet present beneficial effect other than 15 ppm ALA addition. The reason that higher addition level of ALA did not present better effect was unclear until now. In fact, during the heme synthesis, the expression of ALA synthase which is the reaction limiting enzyme is regulated by heme production via a negative feedback mechanism (Doring et al., 1998). The extrinsic ALA supplementation is suggested to bypass such feedback regulation, therefore, results to more heme is produced (Mateo et al., 2006). According to studies conducted by different species, we can approve that the dosages used for piglets may not same with its used for chickens, because of possible species differences in endogenous ALA synthesis. However, the exactly

Table 3. Effect of ALA supplementation on blood characteristics in laying hens[1]

Items	CON	ALA1	ALA2	ALA3	SEM[2]
WBC ($\times 10^5$, No./mm^3)					
0 week	3.82	3.82	4.07	3.73	0.25
8 weeks	4.22	4.36	4.52	4.37	0.30
Difference (0 week-8 weeks)	0.40	0.54	0.45	1.16	0.38
RBC ($\times 10^6$, No./mm^3)					
0 week	2.10	2.14	2.29	2.07	0.10
8 weeks	2.14	2.20	2.30	2.20	0.13
Difference (0 week-8 weeks)	0.04	0.06	0.01	0.13	0.14
Lymphocyte (%)[3]					
0 week	85.2	85.8	79.5	78.0	5.71
8 weeks	67.0	67.0	69.8	81.7	5.72
Difference (0 week-8 weeks)	-18.2	-18.8	-9.75	3.67	11.2
Total protein (g/dl)					
0 week	5.80	5.32	5.20	5.20	0.20
8 weeks	6.20	6.48	6.96	6.28	0.35
Difference (0 week-8 weeks)[4]	0.40	1.16	1.76	1.08	0.33
Albumin (g/dl)					
0 week	2.34	2.18	2.26	2.22	0.08
8 weeks	2.70	2.74	2.80	2.76	0.10
Difference (0 week-8 weeks)	0.36	0.56	0.54	0.54	0.13

[1] Each mean represents ten observations per treatment. Abbreviations: CON, basal diet; ALA1, basal diet with ALA 5 ppm; ALA2, basal diet with ALA 10 ppm; ALA3, basal diet with ALA 15 ppm.
[2] Pooled standard error of the means. [3] Values are presented as percentage of total white blood cell count. [4] Quadratic effect, $p<0.05$.

mechanism of such effect need to be further investigated.

Effects of ALA supplementation on blood characteristics are presented in Table 3. The WBC, RBC, lymphocyte and albumin concentrations were not influenced by the dietary treatments. The difference of total protein concentration between ending and initial of experiment was significantly affected by the ALA supplementation, which the values were greatest in ALA2 treatment, intermediate in ALA1 and ALA3 treatments and lowest in CON treatments (quadratic effect, $p<0.05$).

Total protein and albumin concentrations are generally present protein status in blood. The difference of total protein concentration between beginning and ending of experiment was also higher in ALA2 treatment, whereas albumin concentration did not affected by dietary treatment. Laborde et al. (1995) suggested that the albumin fraction of total protein is more reflective of the long-term protein status, owing to the additional non-albumin fractions of serum total protein. Our data indicated that the non-albumin fraction of serum total protein might be increased in ALA2 treatment, subsequently increased the serum total protein concentration. The non-albumin fraction of total protein consists of globulin and other fractions such as fibrinogen, peptide hormones, enzymes and amino acids (Kaneko, 1989), which has beneficial function for the body. In addition, previous research also suggested that total protein concentration goes up so does that of IgG concentration (Tyler et al., 1996). Therefore, positive effect of ALA addition may also ascribe to influenced total protein level.

Effects of ALA supplementation on egg production and egg weight on laying hens are presented in Table 4. During all the experimental period, supplementation of the basal diet with different levels of ALA did not influenced egg production and egg weight of laying hens. According to current experiment, egg production and egg weight were not affected by ALA supplementation. Park et al. (2004) reported that performance of laying hens was not influenced by different source and level of iron supplementation. In

Table 4. Effects of ALA supplementation on egg production and egg weight in laying hens[1]

Items	CON	ALA1	ALA2	ALA3	SEM[2]
Egg production (%)					
0-4 weeks	92.82	94.11	93.68	92.20	1.76
5-8 weeks	91.59	90.71	92.90	92.44	0.80
Egg weight (g)					
0 week	60.42	61.25	61.72	60.79	0.87
4 weeks	61.54	62.89	63.24	61.42	0.97
8 weeks	61.71	63.22	63.77	62.54	1.06

[1] CON, basal diet; ALA1, basal diet with ALA 5 ppm; ALA2, basal diet with ALA 10 ppm; ALA3, basal diet with ALA 15 ppm.
[2] Pooled standard error of the means.

Table 5. Effect of ALA supplementation on egg shell breaking strength and egg shell thickness in laying hens[1]

Items	CON	ALA1	ALA2	ALA3	SEM[2]
Egg shell breaking strength (kg/cm^2)					
0 week	4.24	4.49	4.37	4.36	0.17
4 weeks	4.55	4.27	4.27	4.58	0.23
8 weeks	4.31	4.31	4.44	4.66	0.15
Difference (0 week-8 weeks)	0.07	-0.18	0.07	0.30	0.09
Egg shell thickness (mm)					
0 week	35.96	35.70	34.96	35.58	0.41
4 weeks	34.93	35.40	34.68	35.22	0.54
8 weeks	35.65	35.42	35.16	35.33	0.42
Difference (0 week-8 weeks)	-0.31	-0.28	0.20	-0.25	0.12
Yolk color unit					
0 week	7.04	7.05	6.99	6.89	0.14
4 weeks	6.72	6.79	6.40	6.32	0.12
8 weeks	7.05	7.01	7.02	6.96	0.11
Difference (0 week-8 weeks)	0.01	-0.04	0.03	0.07	0.06
Egg yolk index					
0 week	0.483	0.486	0.488	0.469	0.007
4 weeks[3]	0.453	0.466	0.466	0.481	0.006
8 weeks[3]	0.465	0.482	0.473	0.496	0.008
Difference (0 week-8 weeks)	-0.018	-0.004	-0.015	0.027	0.004
Haugh unit					
0 week	82.36	80.99	84.77	84.40	1.26
4 weeks	81.20	83.24	85.34	83.53	1.45
8 weeks[3]	77.75	81.56	82.71	87.61	1.84
Difference (0 week-8 weeks)	-4.61	0.57	-2.06	3.21	0.79

[1] Each mean represents 30 observations per treatment. CON, basal diet; ALA1, basal diet with ALA 5 ppm; ALA2, basal diet with ALA 10 ppm; ALA3, basal diet with ALA 15 ppm.
[2] Pooled standard error of the means. [3] Linear effect, $p<0.05$.

addition, current result was also consistent with the study which conducted by Abdallah et al. (1994). In that study, the author suggested that remove supplemental iron or some other kind of minerals (Cu, Zn or Mn) from the laying hen's diets did not affect the egg production and weight of hens. This result indicates that the change of dietary iron concentration may not significant influence egg production of hens. Even though those minerals are necessary for animal's nutrition, due to the body have regulatory mechanisms for mineral absorption and utilization, so such deficient or exceed mineral concentration in diet during a certain period may not present decrease performance criteria of laying hens.

Effects of ALA supplementation on egg quality in laying hens are shown in Table 5. There were no significant differences in egg shell breaking strength, egg shell thickness and yolk color unit among each dietary treatment by ALA supplementation. Egg yolk index were linearly increased at the end of 4 and 8 weeks ($p<0.05$). Similarly, haugh unit was also increased with the increased ALA supplementation levels at the end of 8 weeks (linear effect, $p<0.05$).

Adequate nutrition supply is the basic guarantee to maintain eggshell quality. Gary et al. (1990) suggested that excess trace minerals or vitamins, when fed above the hen's requirement, would not further improve eggshell quality. Current study was in agreement with above mentioned study and also observed no influence of egg shell quality by dietary treatments. The basal diet used in our study was formulated meet or exceed nutrients requirement. In addition, the actual feed consumption of hens was ranged between 105-120 g/d during the experimental period, therefore, the identical egg shell quality may due to hens were already consuming or utilized adequate nutrients.

The hypothesis of this study was improving iron status of laying hens by supplementation of ALA. Therefore, the yolk color was expected to be influenced by dietary treatment because the iron concentration in eggs relate with yolk color. However, present result was out of this anticipation and observed no change on yolk color of laying hens. Ferencik (2000) reported that majority of iron is in blood compared to other organs, therefore, iron status improved in blood may not always indicate same situation in eggs. Egg yolk index and haugh unit values were increased in 15 ppm ALA supplemented treatment, this indicated that the higher storage profile by the ALA supplementation. However, the exactly mechanism for this effect was unclear until and further study should be continued to evaluate and confirm such effect.

IMPLICATIONS

It can be concluded that dietary ALA supplementation at level of 5 ppm can affect iron concentration in serum while higher level (10 or 15 ppm) have some beneficial influences on blood profiles and egg quality. However, it is still necessary to conduct more research of ALA in laying hens, as well as other livestock species, due to the limited data are available in this area.

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