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Fine-Tuning Low-Protein Diets through Vitamin E Supplementation to Avoid Ascites in Broiler Chickens

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ABSTRACT

The present study investigates the effects of Vitamin E (VE) supplement on pulmonary hypertensive response of broilers fed a low-protein diet in a 42-day trial. A total of 180 male broiler chicks (Ross 308) were used in a completely randomized design. Treatments included a Normal Protein Diet (NPD) served as control, a Low-Protein Diet (LPD) with 30 g kg^{-1} of crude protein lower than NPD, and a LPD supplemented with VE (100 mg kg^{-1}). Analyzed protein content of NDP and LPD was 227 and 199 g kg^{-1} in the starter stage and 198 and 169 g kg^{-1} in the grower stage. Growth performance, blood and carcass variables and Lead II of the electrocardiogram (ECG) were recorded. Feed conversion ratio was not significantly changed by treatments. The relative weights of liver, heart, and the right to Total Ventricular weight ratio (RV:TV) as well as the S wave amplitude of ECG were significantly ($P< 0.05$) increased by feeding LPD. However, VE supplementation of LPD significantly ($P< 0.05$) restored these variables to similar ranges observed in NPD. Feeding LPD caused a significant decrease in serum Nitric Oxide (NO) and Uric Acid (UA) concentrations, whereas it caused a significant increase in malondialdehyde (MDA) and Heterophils to Lymphocytes ratio (H:L). Similarly, VE supplement restored these variables to similar levels observed in NPD. In conclusion, oxidative stress was involved in the pathogenesis of ascites in broilers fed with LPD, which could be counteracted by VE supplement.

Keywords: α-Tocopherol, Feed conversion ratio, Growth performance, Pulmonary hypertensive response.

INTRODUCTION

Intensive genetic selection has favored rapid growth (mainly breast muscle) of broiler chickens at the expense of inferior allometric growth of the heart and lungs. As a consequence, an imbalance between oxygen-demanding organs (i.e. muscles) and oxygen-supplying organs (i.e. heart and lungs) has emerged, which leads to Pulmonary Arterial Hypertension syndrome (PAH, also called ascites) (Khajali and Wideman, 2016). Ascites is recognized by accumulation of enormous volume of transudate in the abdominal spaces. The etiology of ascites syndrome is multifactorial in nature. The primary stimulus of ascites syndrome is believed to be hypoxia (Julian, 2007). Hypoxia refers to reduced partial pressure of oxygen, which occurs as the altitude increases. Hence, ascites is the most common metabolic disorder and the major cause of mortality in broiler chickens reared at high altitudes (Izadinia *et al*., 2010). Responses to sustained hypoxia as imposed by high altitude include polycythemia and vascular remodeling characterized by hypertrophy and hyperplasia of the smooth muscle layer in the small arterioles of the lung (Wideman *et al*., 2011).

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Dietary protein content is an important nutritional factor associated with the incidence of ascites. In theory, low-protein diets may appear to be helpful in preventing ascites. This suggestion is based on the assumption that the catabolism of 1g protein in bird's body consumes about 1 L oxygen. Therefore, feeding a low-protein diet may spare oxygen needed for the catabolism of surplus protein and may be attributable to reduced incidence of ascites. However, field and experimental studies revealed opposite results (Maxwell and Robertson, 1998; Buys *et al*., 1998). Behrooj *et al*. (2012) explained that reduced production of uric acid when low-protein diets are fed to chickens is linked to the incidence of ascites. Uric acid is a strong endogenous antioxidant scavenging nitrite, halogenated peroxy radical, and hydroxyl generated radicals (Becker, 1993). In birds, high circulatory level of uric acid contributes to the protection of tissues against Reactive Oxygen Species (ROS) (Machin *et al*., 2004). Research has shown no significant effect of Vitamin E (VE) as an antioxidant on broiler ascites when dietary protein content was formulated to meet commercial norms (Villar-Patiño *et al*., 2002; Bottje *et al*., 1997). However, this might not be true when low-protein diets are fed.

It is noteworthy that chickens are more vulnerable to oxidative stress compared to mammals of comparable size for several reasons. Firstly, chickens have a metabolic rate that is approximately 2 to 2.5 times higher than mammals of comparable body size (Lindstedt and Calde, 1976). This higher rate should increase the quantity of ROS produced from metabolic reactions. Secondly, chickens maintain a high blood sugar concentration that is at least twice as high as that of mammals (Braun and Sweazea, 2008). High blood sugar causes tissue remodeling, which is mediated through the formation of highly reactive methylglyoxal (Kalapos, 1999). The role of methylglyoxal in pathogenesis of ascites in broilers has been addressed by Khajali *et al.* (2011). Last but not least, birds have a body temperature that is about 3°C higher than mammals (Holmes *et al*., 1995). This hyperthermic status may promote non-enzymatic reactions of glucose with proteins leading to formation of advanced glycation end products.

Feeding low-protein diets to poultry has recently been practiced in order to minimize environmental pollution with excessive nitrogen and atmospheric ammonia (Namroud *et al*., 2008; Khajali *et al*., 2008a). However, such nutritional approach cannot be implemented in broiler farms at high altitude regions unless finely-tuned nutritional considerations are put into practice. The objective of the present study was to evaluate the effectiveness of an exogenous antioxidant (vitamin E) to offset reduced production of uric acid in broilers associated with feeding a low-protein diet.

MATERIALS AND METHODS

Birds and Experimental Facility

The experiment was performed in Shahrekord, Iran (2,100 m). The experimental animals were kept, maintained, and treated according to the accepted standards for the humane treatment of animals. The study was accomplished in strict accordance with the recommendations in the Guide for the Care and Use Committee of Shahrekord University, Shahrekord, Iran (ACVC150). Light was provided in 23 L:1 D schedule with intensity of 15 Lux.

A total of 180 day-old male broilers (Ross 308) were randomized across 12 floor pens. Each pen measured 1.8 m^2 (13 birds per pen) and was equipped with a bell drinker and a feed trough. Day-old broiler chicks were grown on a commercial diet until five days of age. Following a six-hour-fast, five-day-old chicks were then allocated to pens so that all pens had equal initial body weights (1,157 g±10 g). Four such pens were allotted to each treatment. The house temperature was maintained at 32±1°C on chicks' arrival

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and reduced to 25±1°C during week 1, 20±1°C in week 2, and 15±1°C onward until the end of trial (42 days of age) as previously described (Khajali and Saedi, 2011). Birds were subjected to 23 hours light and 1 hour dark in entire trial. Birds were allowed free access to feed and water.

Treatments

Two dietary treatments were formulated for the starting (5 to 21 days of age) and growing (21 to 42 days of age) stages according to the NRC (1994) recommendations. A commercial broiler diet with Normal-Protein Content was prepared as control (designated as NPD) to meet or exceed the requirements for all essential amino acids. A Low-Protein Diet (designated as LPD) was also prepared with 30 g kg^{-1} less crude protein compared to the level in NPD. Another similar low-protein diet was also prepared and supplemented with 100 mg kg^{-1} Vitamin E (VE). Analyzed protein content of NDP and LPD was 227 and 199 g kg^{-1} in the starter stage and 198 and 169 g kg^{-1} in the grower stage. All diets had the same level of metabolizable energy and offered in mash form. Potassium carbonate was added to the low-protein diets to equalize the dietary electrolyte balance (Na+K-Cl) among all dietary treatments. The compositions of the experimental starting and growing diets are shown in Table 1.

Prior to the experiment, samples of feed ingredients as well as mixed diets were analyzed for crude protein and amino acid contents. For the determination of amino acid content, duplicate samples of each

Table 1. Compositions of normal- and reduced-protein diets fed to broilers in the starter/grower and finisher periods.

Ingredient (g kg^{-1} unless noted)	Starter/Grower (5-21 d)		Finisher (21-42 d)	
	Normal-protein	Low-protein	Normal-protein	Low-protein
Yellow corn	469.5	546	562	660
Soybean meal	392	335	330	244
Fish meal	25	10	10	5
Soya oil	75	65	61	47
Dicalcium phosphate	15	17	13	14
Oyster shell	14	14.5	15	15.5
Sodium chloride	3.5	3.5	3	3
DL-Methionine	1	2	1	2
L-Lysine HCl	-	-	-	2
Vitamin premix[a]	2.5	2.5	2.5	2.5
Trace mineral premix[b]	2.5	2.5	2.5	2.5
Potassium carbonate	-	2	-	2.5
Analyzed crude protein	227	199	198	169
Analyzed Met+Cys	8.9	8.7	7.3	7.3
Analyzed Lys	13.1	11.0	10.7	10.1
Analyzed Arg	15	12.9	12.6	11.0
Analyzed Thr	9.4	8.4	8.3	7.5
Calculated metabolizable energy (MJ kg^{-1})	13.38	13.38	13.38	13.38
Calculated Na+K-Cl (meq kg^{-1})	235	236	222	222

[a] Provided the following per kg of diet: Vitamin A (trans-retinylvacetate), 1.08 mg; vitamin D3 (cholecalciferol), 0.02 mg; vitamin E (dl-tocopheryl acetate), 7.2 mg; vitamin K3, 1.6 mg; vitamin B1, 0.72 mg; vitamin B2, 3.3 mg; vitamin B3, 0.4 mg; vitamin B6, 1.2 mg; vitamin B12, 0.6 mg; folic acid, 0.5 mg; choline chloride, 200 mg.

[b] Provided the following per kg of diet: Mn (from MnSO4·H2O), 40 mg; Zn (from ZnO), 40 mg; Fe (from FeSO4·7H2O), 20 mg; Cu (from CuSO4·5H2O), 4 mg; I [from Ca(IO3)2·H2O], 0.64mg; Se (from sodium selenite), 0.08 mg.

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ingredient or diet were subjected to 6N HCl and hydrolyzed for 24 hours at 110°C (Andrew and Baldar, 1985). After acid hydrolysis, all samples were analyzed for amino acid content by using an ion-exchange chromatograph (LKB Biochrom 4141; Cambridge, UK). Performic acid oxidation was done to determine sulfur amino acids as well (Moore and Stain, 1963).

Measurements

At 42 days of age, 10 birds per treatment were selected for blood collection. Blood samples (3 mL) were collected from the brachial vein and centrifuged at 2,500×g for 10 minutes to obtain sera. Serum samples were used for the determination of Nitric Oxide (NO), Uric Acid (UA) and MalonDiAldehyde (MDA). Serum NO (nitrate+nitrite) was measured according to Behrooj *et al*. (2012). Serum UA concentration was analyzed according to Fossati *et al*. (1980). Serum MDA concentration, as biomarker of oxidative stress, was assayed by the method of Nair and Turner (1984).

An aliquot of blood was also obtained on glass slides to prepare the blood smear for the determination of differential leukocyte count. The May Grunwald and Giemsa stains were used for staining the smears 3 hours after methyl alcohol fixation (Lucas and Jamroz, 1961). One hundred leukocytes, including granular (heterophils) and nongranular (lymphocytes) were enumerated and the Heterophil to Lymphocyte ratio (H:L) was calculated. All chemical reagents were obtained from Sigma–Aldrich Co. (Sigma–Aldrich Co., St. Louis, MO, USA).

The birds were then slaughtered to obtain liver and heart weights. Hearts were dissected to obtain right-to-total ventricular weight ratio.

Electrocardiographic Recording

Ten chicks per treatment were randomly selected at day 40 to record ElectroCardioGrams (ECG). The electrocardiograph (Kenz ECG 110, Suzuken Japan CO. LTD) was standardized at 10 mm= 1 mV with a chart speed of 50 mm s^{-1} (Yousefi *et al*., 2013). Leads II of ECG was recorded for every chicken and the amplitude of the T, R and S waves were measured.

Statistical Analysis

Results were analyzed by GLM procedure of SAS (2007) software in a completely randomized design. The statistical model used for growth performance data (experimental units were pens of 13 birds each) was $Y_{ij}= \mu+T_i+e_{ij}$. For blood (H:L), sera (NO, UA, and MDA) and organ (liver and heart percentages and RV:TV) data (experimental units individual birds; n= 10), the model was $Y_{ijk}= \mu+T_i+eij+\varepsilon_{ijk}$. In these models, Y_{ij} and Y_{ijk} are observations; μ is the general location parameter (i.e., the mean); T_i is the effect for being in Treatment *i*; e_{ij} is random error; and ε_{ijk} is subsampling error. Means were separated by Duncan's multiple range with the probability of 95%.

RESULTS

Table 2 shows growth performance of broiler chickens fed with a low-protein diet and VE supplementation. Feed intake and weight gain were not significantly altered by feeding a low-protein diet. However, these variables were significantly altered in VE-supplemented Low-Protein Diet compared to the control (NPD). Feed conversion ratio did not change by treatments.

Table 3 depicts carcass variables including the relative weights of liver, heart, and abdominal fat as well as *RV:TV* and ascites mortality. The relative weights of liver, heart and *RV:TV* were significantly ($P< 0.05$) higher in birds fed with LPD compared to those that received NPD. Supplementing LPD with VE restored the situation so that no difference was observed for these

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Table 2. Effect of dietary protein content and Vitamin E (VE) supplement on performance of broiler chickens from 5 to 42 days of age (means±SE).

Variables	NPD [a]	LPD [b]	LPD+VE	*P*-value
Total weight gain (g b^{-1})	2351 [a]±54.7	2235 [ab]±38.5	2122 [b]±40.7	0.018
Total feed intake (g b^{-1})	4414 [a]±94.9	4242 [ab]±71.1	3987 [b]±89.7	0.019
Feed conversion ratio	1.87±0.012	1.90±0.014	1.88±0.015	0.481

[a] Normal-Protein Diet; [b] Low-Protein Diet; Vitamin E was added at the top of reduced-protein diet at 100 mg kg^{-1}. Superscripts in the same row with different letters are significantly different (P< 0.05).

Table 3. Effect of dietary protein content and Vitamin E (VE) supplement on internal organs and cumulative ascites mortality in broiler chickens measured at 42 days of age (means±SE).[a]

Variables	NPD [a]	LPD [b]	LPD+VE	*P*-value
Liver (% BW)	2.38^{b}±0.06	2.87^{a}±0.19	2.21^{b}±0.08	0.008
Heart (% BW)	0.58^{b}±0.18	0.72^{a}±0.041	0.62^{b} ±0.033	0.013
RV:TV	0.24^{b}±0.013	0.30^{a}±0.015	0.25^{b} ±0.013	0.018
Ascites mortality (%)	20.1^{ab}±4.24	28.3^{a}±3.33	14.5^{b}±1.23	0.039

[a] Normal-Protein Diet; [b] Low-Protein Diet; Vitamin E: Use at 100 mg kg^{-1}. Each mean represents values of 10 replicates and means in the same row with different letters are significantly different (P< 0.05).

parameters in comparison with NPD. Cumulative ascites mortality was significantly reduced by VE supplementation of LPD (P< 0.05).

Effects of dietary treatments on serum and blood variables are presented in Table 4. Serum concentrations of NO and UA in LPD were significantly (P< 0.05) lower than NPD. The levels of NO and UA tended to increase by supplementing LPD. Circulatory level of MDA in birds fed with LPD was significantly (P< 0.05) higher than birds fed with NPD. VE supplementation of LPD decreased (P< 0.05) circulatory MDA level to similar level observed in NPD. A significant elevation in *H:L* (P< 0.05) was also observed in broilers fed with LPD when compared to the control (NPD). However, VE supplementation of LPD significantly (P< 0.05) reduced *H:L*.

There was a significant negative increase in S-wave amplitude of birds fed LPD compared to NPD (Table 5). Vitamin E supplement restored the situation so that it

Table 4. Effect of dietary protein content and Vitamin E (VE) supplement on blood and serum variables in broiler chickens measured at 42 days of age (means±SE).

Variables	NPD[a]	LPD[b]	LPD+VE	*P*-value
Serum nitric oxide (µmol)	15.60^{a}±0.73	9.91^{b}±1.31	12.33^{b}±0.85	0.0027
Serum malondialdehyde (µmol)	2.44^{b}±0.44	3.87^{a}±0.51	2.21^{b}±0.42	0.041
Serum uric acid (mg dl^{-1})	6.23^{a}±0.43	4.46^{b}±0.44	5.00^{b}±0.21	0.010
H:L	0.61^{b}±0.030	0.86^{a}±0.075	0.58^{b}±0.034	0.0015

[a] Normal-Protein Diet; LPD: Low-Protein Diet; Vitamin E: Use at 100 mg kg^{-1}. Each mean represents values of 10 replicates and means in the same row with different letters are significantly different (P< 0.05).

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Table 5. Effect of dietary protein content and Vitamin E (VE) supplement on electrocardiographic (lead II) parameters measured at 42 days of age (means±SE).

Variables	NPD [a]	LPD [b]	LPD+VE	*P*-value
R wave(mV)	0.21±0.035	0.22±0.035	0.25±0.026	0.311
S wave(mV)	-0.30[b]±0.016	-0.38[a]±0.019	-0.28[b]±0.018	0.011
T wave (mV)	0.15±0.018	0.18±0.035	0.13±0.017	0.223

[a] Normal-Protein Diet; [b] Low-Protein Diet; Vitamin E: Use at 100 mg kg^{-1}. Each means for cardiac/electrocardiographic represents values of 10 replicates and means in the same row with different letters are significantly different (P< 0.05).

had no significant difference with NPD. There were no significant differences for R and T wave amplitudes among the treatments (Table 5).

DISCUSSION

Live performance of birds fed LPD did not significantly differ from those that received NPD. Widyaratne and Drew (2011) indicated that low-protein diets could support growth performance of broiler chickens similar to that of high-protein diet as long as highly digestible feed ingredients were used. Birds on LPD supplemented with VE, however, showed lower weight gain and feed intake when compared to NPD. The reason is not clear how VE might have deteriorated the growth response when added to a low-protein diet.

The relative weights of liver and heart significantly increased by feeding LPD. The Right to Total Ventricular weight ratio (RV:TV) was also increased when broilers fed with LPD were compared to NPD. *RV:TV* is indicator of pulmonary hypertension and ascites (Izadinia *et al*., 2010). *RV:TV* values greater than 0.25 indicate pulmonary hypertension and values greater than 0.3 indicate pre-ascitic condition. It is clear that birds fed the low-protein diet were in pre-ascitic condition. Supplementing VE to the low-protein diet counterbalanced the response so that there was a significant difference between LPD with or without VE supplement with respect to *RV:TV* and ascites mortality. Supplement of VE was previously reported to prevent further increases in *RV:TV* in broilers reared under low temperature (Aksit *et al*., 2008). Bottje *et al*. (1997) showed that supplementing diet with VE resulted in lower *RV:TV*, suggesting an ameliorating effect of events leading to development of ascites.

Switching from NPD to LPD caused a significant decrease in serum NO. This finding can be explained in part by a decrease in dietary Arginine (Arg) content (see Table 1). In fact, Arg is the precursor of NO synthesis. Nitric oxide is a potent vasodilator that opposes the onset of pulmonary hypertension in broiler chickens (Khajali and Wideman, 2010; Khajali *et al*., 2014). This finding is in line with higher *RV:TV* in LPD compared to the control. It is worthy to note that Arg requirements of birds raised at high altitude are significantly higher than the recommended values of NRC (Khajali *et al*., 2013). Supplement of VE was not able to restore NO to the level observed in NPD. Switching from NPD to LPD was attributed to increased lipid peroxidation as manifested by a significant rise in MDA. Oxidative stress has been implicated in the pathogenesis of pulmonary hypertension (Nain *et al*., 2008; Khajali and Wideman, 2016). It has been shown that superoxide and NO reacts very rapidly in a third-order reaction that is approximately 3 times faster than the dismutation of superoxide by superoxide dismutases. Thus, increased generation of superoxide in the vascular wall might have inhibited the physiological functions of NO (Nain *et al*.,

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2008). In this experiment, elevated serum MDA level reflects lipid peroxidation due to oxidative stress, which has been significantly offset by adding VE to LPD. In agreement with our results, Nain *et al.* (2008) reported the modulated effect of VE on lipid peroxidation and MDA level in broilers exposed to cold temperature.

The *H:L* ratio is a reliable indicator of stress in birds (Gross and Siegel, 1983; Khajali *et al*., 2008b). The higher the *H:L* value, the more severe the stress. It is evident that birds on LPD were exposed to more stress than their counterparts on NPD. Supplementation of LPD with VE significantly reduced *H:L* and alleviated the stress. In this regard, it has been reported that VE supplement alleviate the adverse effects of chronic heat stress in poultry (Bollengier-Lee *et al*., 1998).

The S-wave amplitude of electrocardiogram was significantly increased by feeding the low-protein diet. An increase in S-wave amplitude reflects the hypertrophy of the right ventricular wall that can be directly related to increased pulmonary arterial pressure (Yousefi *et al*., 2013). This finding is in good accordance with higher *RV:TV* and ascites mortality in LPD group relative to the control. Interestingly, VE supplementation of LPD has been able to avoid such abnormal increases in S-wave amplitude with concomitant decline in *RV:TV* and ascites mortality.

CONCLUSIONS

In conclusion, feeding a low-protein diet is associated with Right Ventricular Hypertrophy (RVH) in broiler chickens. Supplementing vitamin E to a low-protein diet effectively counterbalances the adverse impact of low-protein diets on RVH.

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بهینه سازی جیره های کم پروتئین با مکمل سازی ویتامین E برای پیشگیری از وقوع آسیت در مرغ های گوشتی

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چکیده

در پژوهش حاضر تاثیر افزودن ویتامین E در جیره‌های کم پروتئین بر وقوع فشار خون ریوی جوجه‌های گوشتی بررسی شد. برای این منظور از ۱۸۰ قطعه جوجه خروس گوشتی (راس ۳۰۸) در ۳ تیمار در قالب طرح کاملاً تصادفی استفاده شد. تیمارها شامل یک جیره با سطح پروتئین نرمال و یک جیره کم پروتئین با ۳۰ گرم در کیلوگرم پروتئین خام کمتر نسبت به جیره با پروتئین نرمال بود. یک جیره کم پروتئین مشابه دیگر نیز با افزودن سطح mg/kg ۱۰۰ ویتامین E تهیه شد. در این آزمایش علاوه بر عملکرد رشد، داده های سرمی و خونی و الکتروکاردیوگرام اشتقاق II نیز جمع آوری و آنالیز شد. نتایج نشان داد که ضریب تبدیل خوراک تحت تاثیر تیمارها قرار نگرفت. وزن نسبی کبد، قلب و نسبت بطن راست به مجموع بطن‌ها و همچنین امواج منفی S در الکتروکاردیوگرام به طور معنی‌داری در گروه کم پروتئین بیشتر از شاهد بود. افزودن ویتامین E به جیره کم پروتئین سبب کاهش معنی‌دار این متغیرها شد، به طوری که اختلافی با گروه شاهد مشاهده نشد. تغذیه جیره‌های کم پروتئین سبب کاهش معنی‌دار غلظت سرمی نیتریک اکساید، اسید اوریک و نسبت هتروفیل به لنفوسیت شد. افزودن ویتامین E به جیره‌های کم پروتئین سبب بازگشت سطوح این فراسنجه‌ها به سطوح مشابه آن در گروه پروتئین نرمال شد. به طور کلی، نتایج این آزمایش تایید کننده اثر تنش اکسیداتیو ناشی از کاهش پروتئین خوراک در افزایش وقوع آسیت می باشد و همچنین تاثیر مثبت ویتامین Eدر پیشگیری از هایپرتروفی بطن راست و مرگ و میر ناشی از آسیت تحت این شرایط را تایید نمود.

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