Dietary L-Carnitine Supplement Counteracts Pulmonary Hypertensive Response in Broiler Chickens Fed Reduced-Protein Diets and Subjected to Cool Condition and Hypobaric Hypoxia

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The present study was carried out to evaluate the effects of supplementing reduced-protein diets with L-carnitine on growth performance and occurrence of pulmonary arterial hypertension (PAH) syndrome in broiler chickens reared at high altitude. A total of 156 day-old male broilers (Ross 308) were assigned to three dietary treatments and reared up to 42 days of age. A normal-protein diet (NPD) was formulated according to the National Research Council (1994) and served as control. A reduced-protein diet (RPD) was also prepared to contain 3% less protein than that of the NPD. An additional RPD diet was prepared by supplementing L-carnitine (LC) at 100 mg/kg to the RPD. Results showed significant improvements in feed:gain and carcass yield in birds fed on RPD when supplemented with LC. The proportions of liver, heart, and abdominal fat pad relative to body weight and the right ventricular weight ratio (RV:TV) were significantly higher in birds fed on the RDP than those of the control fed the NPD. Electrocardiogram (ECG) measurements recorded from lead II supported the development of PAH in birds fed RPD as evidenced by deep S waves. Supplementing LC to the RPD significantly reduced the liver weight, abdominal fat deposition, and RV:TV, which reflected in reduced S waves. Feeding broilers with the RPD significantly reduced serum concentrations of nitric oxide (NO) and uric acid (UA). However, supplementation of the RPD with LC significantly increased the serum concentrations of NO. In conclusion, feeding reduced-protein diets to broilers reared at hypobaric hypoxia increases their susceptibility to pulmonary hypertension. Dietary LC supplementation of reduced-protein diets had beneficial effects in preventing PAH mortality mainly through enhancing blood NO concentration.

Key words: broiler ascites, carnitine, lipogenesis, nitric oxide

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Introduction

L-carnitine (LC) is the main component of carrier-mediated enzymatic shuttle in the inner membrane of mitochondria, which transport long-chain fatty acids from the cytoplasm to the mitochondrial matrix. The carnitine palmitoyltransferase is the enzyme responsible for this shuttle mechanism. Long-chain fatty acids entered the mitochondrial matrix are subjected to β-oxidation process, which is the main lipolytic pathway. Xu et al. (2003) investigated the effect of dietary LC supplement (0 to 100 mg/kg) on growth performance and fat metabolism in broilers. They observed a decrease in abdominal fat and muscle fat contents when LC was supplemented to diets at greater than 25 mg/kg. Lien and Horng (2001) indicated that carnitine palmitoyl transferase activity in the carnitine-supplemented group was significantly ($P<0.05$) higher in birds received dietary LC supplement compared to the control. However, LC supplement had no effect on the activities of fatty acid β-oxidation enzymes. Rodehutscord et al. (2002) reported that LC supplement (80 mg/kg) did not significantly improve the efficiencies of energy and protein utilization, suggesting LC may have other important biological impacts. Yousefi et al. (2013) indicated that LC supplement (100 mg/kg) increased circulatory level of nitric oxide (NO), an important cellular signaling molecule involved in many physiological processes. Nitric oxide is also a powerful vasodilator that prevents pulmonary hypertension (Izadinia et al., 2010; Khajali et al., 2011a). Besides, LC has been shown to play an antioxidant role in animal metabolism (Lee et al., 2014).

Research has shown that mortality from pulmonary arterial hypertension (PAH) syndrome was significantly increased in broiler chickens fed on reduced-protein diets and reared at...
Material and Method

Birds and Experimental Facility

The experiment was carried out in an area with an altitude of 2,100 m above sea level. The experimental animals were kept, maintained and treated in strict accordance with the recommendations in the Guide for the Care and Use Committee of Shahrekord University.

A total of 156 day-old male broilers (Ross 308) were randomized across 12 floor pens. Each pen measured 1.8 m² (13 birds per pen) and was equipped with a bell drinker and a feed trough. All chicks were raised on a commercial starter diet until five days of age. Following a 6h fast, chicks were transferred to new tubes and subjected to Griess reaction. Griess reagent 2 (N-naphthylethylenediamine dihydrochloride in water) was then dispensed to all samples and the absorbance of NO₂⁻ was measured at 540 nm within 10 min by a spectrophotometer (Corning 480, Corninig, New York, NY, USA).

Serum UA concentration was analyzed colorimetrically according to Fossati et al. (1980) using 3,5-dichloro-2-hydroxybenzene sulfonic acid/4-aminophenazone chromogenic system in the presence of horseradish peroxidase and uricase. The red color formation was then measured at 520 nm. MDA concentration in the serum samples was measured as an index of lipid peroxidation by the TBARS (thiobarbituric acid reactive substances) method (Nair and Turner, 1984). MDA is formed as a result of lipid peroxidation and reacts with thiobarbituric acid under high temperature (90–100°C) and acidic condition. The reaction yields a pink MDA-TBA adduct. The colored complex was measured by a spectrophotometer at 535 nm.

Samples of blood were also collected in microhematocrit tubes for measuring hematocrit. An aliquot of blood was also obtained on glass slides to prepare the blood smear for the determination of differential leukocyte count. May Grunwald-Giemsa staining was used for blood collection. Blood samples (3 mL) were collected from the brachial vein and centrifuged at 2,500 × g for 10 min 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). This assay was based on the reduction of nitrate to nitrite by cadmium. Serum samples were deproteinised by adding 75 mmol ZnSO₄ and 55 mmol NaOH. Samples were then centrifuged and the resulting supernatants were diluted with glycine buffer (45 g/L; pH 9.7). Freshly activated cadmium granules (~ 2 g rinsed three times in deionized water and swirled in 5 mmol CuSO₄ in glycine-NaOH buffer 5 g/L; pH 9.7) were added to samples. Upon continuous stirring for 10 min, the samples were transferred to new tubes and subjected to Griess reaction. Griess reagent 2 (N-naphthylethylenediamine dihydrochloride in water) was then dispensed to all samples and the absorbance of NO₂⁻ was measured at 540 nm within 10 min by a spectrophotometer (Corning 480, Corninig, New York, NY, USA).

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At the end of trial (day 42), 8 additional birds per treatment were euthanized for carcass processing. Data obtained...
at processing included hot eviscerated carcass weight, breast weight, liver weight and abdominal fat deposition. The hearts were also harvested and the ventricles were dissected and weighed to calculate the right-to-total ventricular weight ratio (RV:TV ratio). Mortality from PAH was checked daily and identified by RV:TV values greater than 0.299 (Walton et al., 2001).

Electrocardiographic Recording

Neither sedative nor anesthetic material was used for the ECG recordings. The chickens were placed in a ventral (sternal upright) recumbent position on a plastic-covered table. In easily stressed birds, the head was covered with a surgical cloth to relieve stress during handling. Four alligator clip electrodes were connected to the cranial area of the skin fold in the angle between the arm and forearm of the left and right wings (propatagium) and to the skin on the left and right skin over the cranial cnemial crest. The use of alligator clips without sharp teeth avoided any damage to the feathers and skin. All procedures took place in an isolated room to minimize the stress to the birds. Eight chicks per treatment were randomly selected at day 40 and electrocardiograms (ECG) were recorded. The electrocardiograph (Kenz ECG 110, SUZUKEN CO., LTD. Nagoya, Japan) was standardized at 10 mm = 1 mV with a chart speed of 50 mm/s (Hassanpour et al., 2009; Yousefi et al., 2013). Leads II 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. For live performance data, the individual experimental unit was the pen of birds and a completely randomized analysis of variance was used to compare the treatment means in a model as \( Y_{ij} = \mu + T_i + e_{ij} \). For blood (haematocrit, H:L), sera (NO, UA, and MDA) and carcass (carcass yield, breast yield, liver and heart percentages, abdominal fat deposition and RV:TV) data, where there was sampling within pens, data were subjected to a nested design and the model was \( Y_{ijk} = \mu + T_i + e_{ij} + e_{ijk} \). In these models, \( Y_{ij} \) and \( Y_{ijk} \) are observations; \( \mu \) 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 \( e_{ijk} \) is subsampling error. The probability level of \( P<0.05 \) was chosen to present significant differences for all data. Means were separated by the Duncan’s multiple range test.

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

| Ingredient (g/kg unless noted) | Starter/grower (5–21d) | Finisher (21–42d) |
|-------------------------------|------------------------|-------------------|
|                               | Normal-protein | Reduced-protein | Normal-protein | Reduced-protein |
| Yellow corn                   | 469           | 548            | 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                  | –             | –              | –            | –             |
| Vitamin premix\(^1\)          | 2.5           | 2.5            | 2.5          | 2.5           |
| Trace mineral premix\(^2\)    | 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) | 13.38       | 13.38          | 13.38        | 13.38         |
| Calculated Na + K-Cl (meq/kg) | 235           | 236            | 222          | 222           |

\(^1\)Provided the following per kg of diet: vitamin A (trans-retinylvacetate), 1.08 mg; vitamin D\(_3\) (cholecalciferol), 0.02 mg; vitamin E (d-l-tocopheryl acetate), 7.2 mg; vitamin K\(_3\), 1.6 mg; vitamin B\(_1\), 0.72 mg; vitamin B\(_2\), 3.3 mg; vitamin B\(_3\), 0.4 mg; vitamin B\(_6\), 1.2 mg; vitamin B\(_12\), 0.6 mg; folic acid, 0.5 mg; choline chloride, 200 mg.

\(^2\)Provided the following per kg of diet: Mn (from MnSO\(_4\)·H\(_2\)O), 40 mg; Zn (from ZnO), 40 mg; Fe (from FeSO\(_4\)·7H\(_2\)O), 20 mg; Cu (from CuSO\(_4\)·5H\(_2\)O), 4 mg; I (from Ca(IO\(_3\))\(_2\)·H\(_2\)O), 0.64 mg; Se (from sodium selenite), 0.08 mg. L-carnitine was added at the top of reduced-protein diet at 100 mg/kg.
Table 2. Effect of L-carnitine supplementation of reduced-protein diets on cardiac/electrocardiographic (lead II) wave amplitudes and PAH mortality

| Variables               | NPD           | RPD           | RPD + L-carnitine | P-value |
|-------------------------|---------------|---------------|-------------------|---------|
| R wave (mV)             | 0.21 ± 0.035  | 0.22 ± 0.035  | 0.23 ± 0.026      | 0.8974  |
| S wave (mV)             | −0.30 ± 0.016 | −0.38 ± 0.019 | −0.31 ± 0.030     | 0.0308  |
| T wave (mV)             | 0.15 ± 0.018  | 0.18 ± 0.035  | 0.15 ± 0.017      | 0.6940  |
| PAH mortality (%)       | 20.1 ± 4.24   | 28.3 ± 3.33   | 17.5 ± 0.39       | 0.0460  |
| RV:TV in PAH birds      | 0.39 ± 0.023  | 0.41 ± 0.019  | 0.39 ± 0.018      | 0.2015  |

Each mean represents values from 8 replicates.
NPD: normal-protein diet; RPD: reduced-protein diet; L-carnitine: used at 100 mg/kg
PAH: pulmonary arterial hypertension; RV:TV: right to total ventricular weight ratio

Results

There was a significant ($P=0.0308$) negative increase in S wave amplitude of birds fed RPD compared with NPD (Table 2). L-carnitine supplementation restored the situation so that it had no significant difference with NPD. There were no significant differences for R and T wave amplitudes among the treatments. Cumulative PAH mortality was significantly reduced by LC supplementation (Table 2).

Table 3 depicts carcass variables measured at the end of the experiment (42 days). Feeding RPD was associated with a significant ($P=0.0467$) reduction in carcass yield when compared to NPD. Supplementing RPD with LC restored the situation so that no difference was observed for carcass yield in comparison with NPD. The proportional weights of liver ($P=0.0108$), heart ($P=0.0333$), abdominal fat ($P=0.0252$) and the RV:TV ($P=0.0178$) ratio were significantly higher in birds fed on RPD compared to those fed on NPD. Supplementing LC to RPD restored the situation.

Effects of dietary treatments on serum and blood variables are presented in Table 4. Circulatory level of NO in birds fed RPD was significantly ($P=0.0013$) lower than birds fed NPD. The level of NO, however, significantly ($P=0.0013$) increased by supplementation of RPD with LC though it was still lower than NPD. Serum uric acid concentration significantly reduced ($P=0.0057$) in birds fed RPD with or without LC supplement when compared to NPD. Though hematocrit did not influence by dietary protein content and LC supplementation, the H:L ratio was significantly ($P=0.0034$) increased in birds fed RPD when compared to those fed on NPD. Supplementing RPD with LC reduced the H:L ratio to the same level observed in NPD. No significant difference was observed among dietary treatments in terms of MDA.

Body weight gain, feed intake and feed:gain throughout the trial were not significantly different between NPD and RPD. However, LC supplementation of RPD significantly ($P=0.05$) improved feed:gain (Table 5).

Discussion

There was no significant change in growth performance of broilers as a result of reduction in dietary protein content. This finding is in agreement with previous research (Laudadio et al., 2012). Supposedly, if reduced-protein diets are adequately supplemented with essential amino acids to meet the nutritional requirements of birds, growth performance can be comparable to those fed conventional (normal-protein) diets. Supplemental LC had no significant effect on body weight gain and feed intake but significantly improved feed:gain, which is in line with previous reports (Rabie and Szilagyi, 1998). Moreover, carcass yield significantly increased by LC supplementation of RDP. Though studies on broilers reared at normoxic condition and fed normal-protein...
diets indicated that LC did not influence feed efficiency and carcass yield of broiler chickens (Buyse et al., 2001; Lien and Horng, 2001), this dietary supplement improves feed efficiency and carcass yield for broilers subjected to hypoxic condition and fed on reduced-protein diets.

Feeding the RPD caused a significant increase in relative weight of liver and abdominal fat deposition when compared to the NPD. This finding demonstrates intensive hepatic lipogenesis occurred by feeding reduced-protein diets. In line with our observations, Rosebrough et al. (1999) showed that feeding reduced-protein diets intensified liver lipogenesis. In birds, the liver is the principal site of lipogenesis whereas adipose tissues account for a very limited lipogenesis (Stevens, 1996). Intensive lipogenesis can be reflected in increased abdominal fat deposition (Hood, 1984; Rosebrough et al., 1999).

Feeding reduced-protein diet significantly increased the relative weight of heart and the RV:TV ratio. The RV:TV ratio is indicative of PAH (Saedi and Khajali, 2010; Wideman et al., 2011; Ahmadipour et al., 2015). It is evident that chickens fed on RPD were expressing complications associated with PAH. In fact, intensive lipogenesis incurs high oxygen demand and push the right ventricle to pump more blood to the lungs for oxygenation. Subsequent overwork of heart especially the right ventricle causes right ventricular hypertrophy, which is manifested as increased RV:TV. Interestingly, LC supplement significantly reduced RV:TV in birds fed on RPD by suppressing lipogenesis and increasing circulatory level of NO. Nitric oxide is a potent vasodilator that opposes the onset of PAH in broiler chickens (Khajali and Wideman, 2010; Khajali et al., 2011b). In agreement with our study, Tan et al. (2008) reported a lower RV:TV in broilers fed with LC. Several mechanisms have been explained the role of LC in increased production of NO. Erbas et al. (2007) indicated that LC increased NO through the reduction in the activity of arginase and elevation in the activity of NO synthase. Another study by Sharifi et al. (2009) showed that LC could reduce the activity of angiotensin converting enzyme, which resulted in higher NO production. Recent research revealed that LC could increase NO production through activation of phosphatidylinositol 3-kinase and subsequent stimulation of endothelial nitric oxide synthase (eNOS) (Ning and Zhao, 2013). In line with our finding, Yousefi et al. (2013) indicated that LC supplement (100 mg/kg) increased circulatory level of NO. The changes observed herein have been reflected in ECG of birds in this study. Electrocardiogram indicates that the amplitude of the S waves is significantly reduced by administration of L-carnitine to the broilers fed on RDP. Kirby et al. (1999) reported a relatively high correlation between S wave amplitude and RV:TV. Negatively lower S wave amplitudes in broilers received L-carnitine suggest a lower rate of right ventricular hypertrophy and dilation. Altogether, these observations explain a significant reduced in mortality from PAH observed in birds fed LC-supplemented RPD. Olkowski et al. (2007) reported that the LC content of heart in broilers with heart failure was lower than in healthy broilers. They reported that lower level of myocardial LC

### Table 4. Effect of L-carnitine supplementation of reduced-protein diets on blood and serum variables in broiler chickens

| Variables                        | NPD             | RPD             | RPD + L-carnitine | P-value |
|----------------------------------|-----------------|-----------------|-------------------|--------|
| Serum nitric oxide (μmol)        | 15.60±0.73      | 9.91±1.31       | 12.64±0.90        | 0.0013 |
| Serum uric acid (mM/L)           | 0.37±0.026      | 0.27±0.015      | 0.30±0.017        | 0.0057 |
| Serum malondialdehyde (μmol)    | 2.44±0.44       | 3.87±0.51       | 2.41±0.48         | 0.1114 |
| Hematocrit (%)                  | 40.6±1.16       | 43.8±2.65       | 40.0±1.07         | 0.4310 |
| H:L                              | 0.61±0.030      | 0.86±0.075      | 0.60±0.074        | 0.0034 |

Each mean represents values from 8 replicates.
NPD: normal-protein diet; RPD: reduced-protein diet; L-carnitine: used at 100 mg/kg
H:L: heterophils to Lymphocytes

### Table 5. Effect of L-carnitine supplementation of reduced-protein diets on performance of broiler chickens

| Variables          | NPD            | RPD            | RPD + L-carnitine | P-value |
|--------------------|----------------|----------------|-------------------|--------|
| Total weight gain | 2351 ±54.7     | 2235 ±38.5     | 2252 ±69.4        | 0.3282 |
| (g/b)              |                |                |                   |        |
| Total feed intake  | 4414 ±94.9     | 4242 ±71.1     | 4150 ±104.0       | 0.1705 |
| (g/b)              |                |                |                   |        |
| Feed:gain          | 1.87±0.012     | 1.90±0.014     | 1.84±0.013        | 0.0500 |

Each mean represents values from 4 replicates.
NPD: normal-protein diet; RPD: reduced-protein diet; L-carnitine was added at the top of RPD at 100 mg/kg.
was attributed to deterioration of heart function (Olkowski et al., 2007).

Plasma UA was significantly reduced in the chickens fed RPD compared to those fed NPD. Blood UA contributes to the protection of tissues against reactive oxygen species (ROS) (Machin et al., 2004). Reduced production of UA in birds fed on reduced-protein diets was reported to contribute to the development of PAH (Behrooj et al., 2012). No significant effect on uric acid was noted due to adding LC to RDP group. Hematocrit and MDA level were not significantly changed by feeding reduced-CP diets and LC supplementation. Heterophils to lymphocytes ratio (H:L) was significantly increased by feeding RPD. The H:L ratio is an index of stress in the chicken (Khajali et al., 2011a) and its higher value suggests birds fed on RPD are under stress presumably because they are suffering from pulmonary hypertension. Supplementing LC to RPD significantly reduced the H:L ratio and this suggests therapeutic effects of LC in amelioration of PAH.

Conclusion

In general, feeding reduced-protein diets to broilers reared at hypobaric hypoxia increases their susceptibility to pulmonary arterial hypertension and causes PAH mortality. Dietary L-carnitine supplementation of reduced-protein diets has beneficial effects in counteracting the problem mainly by enhancing blood NO concentration.

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