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Supplemental L-arginine Modulates Developmental Pulmonary Hypertension in Broiler Chickens Fed Reduced-Protein Diets and Reared at High Altitude

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Abstract

This experiment was conducted to evaluate the effects of supplemental L-arginine (ARG) in reduced-protein diets on cardiopulmonary performance and intestinal morphology in the broilers reared at high altitude. A total of 156 day-old male broilers were randomly assigned to 3 treatments and 4 replicates of 13 chicks and reared up to 42 days of age. Treatment groups were designed as a normal-protein diet (NPD), a reduced-protein diet (RPD) with 30 g/Kg less crude protein compared to the NPD, and a reduced-protein diet plus 4 g/Kg L-arginine (RPD + ARG). There were no significant differences among dietary treatments for intestinal morphology and weight gain. Feed conversion ratio was improved in the chickens fed RPD + ARG compared to those fed RPD alone. The right to total ventricular weight ratio (RV:TV) was significantly increased in the chickens fed RPD when compared to those fed NPD or RPD + ARG. Serum nitric oxide and amplitude of the S waves of electrocardiogram significantly declined by reducing dietary protein content. Relative expression of endothelin-1 (ET-1) gene was higher in the heart and lungs of chicks fed RPD than those fed NPD and it was off set when ARG supplemented to RPD ($P<0.05$). In conclusion, supplementing reduced-protein diets with ARG would be an effective strategy to prevent the development of pulmonary hypertension by increase in nitric oxide, and decrease in RV:TV and ET-1 gene expression.

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Introduction

In recent years, nutritional approaches in feed formulation for various classes of poultry have focused on feeding reduced-protein diets to minimize N excretion to the environment (Aletor *et al*., 2000; Khajali *et al*., 2008) and to reduce feed cost of production (Kamran *et al*., 2008). However, new studies have reported that application of this nutritional strategy at high altitudes reduce optimal performance and predispose broilers to pulmonary hypertension syndrome (PHS), which in part is attributed to the decline in dietary ARG content in such diets (Behrooj *et al*., 2012).

Meat-type (broiler) chickens are highly susceptible to PHS due to intensive genetic selection for either growth rate or feed conversion efficiency, which incur high demands on metabolic rate and oxygen consumption. In such situation, cardiac output must be overloaded to increase pulmonary arterial pressure (PAP) to avoid hypoxic condition. However, sustained elevation of PAP causes hypertrophy and subsequent dilation of the right ventricle (RV). Meanwhile, an imbalance between cardiac output and anatomical capacity of the pulmonary vasculature to accommodate ever-increasing rates of blood flow result in an inappropriately elevated tone (degree of constriction) maintained by the pulmonary arterioles (Hassanzadeh *et al*., 2008; Ruiz-Feria, 2009). All factors that increase the pulmonary vascular resistance can initiate or accelerate the pathophysiological progression leading to PHS such as hypoxia, adrenergic neurotransmitters, thromboxane A2, endothelin-1, 5-hydroxytryptamine, respiratory damage or disease. The key pulmonary vasodilator for broilers is nitric oxide (NO), which is synthesized from ARG (Tan *et al*., 2007). Deficiency in dietary ARG and resulting decline in NO leads to pulmonary arterial vasoconstriction, pulmonary hypertension, and PHS (Hassanpour *et al*., 2009a; Khajali and Wideman, 2010). Another critical factor in the pathogenesis of PHS is high level of ET-1 which is the most potent vasoconstrictor produced by the cardiovascular system (Hassanpour *et al*., 2011). Previous studies have implied to existence of local ET system in the heart and lung which considerably involve in the cardiopulmonary system. ET appears to exert differential effects on normal and failing myocardium, exerting a positive inotropic effect in the normal heart and a negative inotropic effect in the failing heart (Shah, 2007; Hassanpour *et al*., 2010). Supplementing L-arginine to broiler diets facilitated pulmonary vasodilatation in response to large increases in blood flow (Wideman *et al*., 1996) and modulated the pulmonary hypertension (Ruiz-Feria, 2009; Khajali *et al*., 2011; Saki *et al*., 2013; Khajali *et al*., 2014). Diets supplemented with ARG have shown to increase plasma NO levels and increase growth performance and attenuate symptoms of pulmonary hypertension in broilers exposed to combined challenges of cool temperature and hypobaric hypoxia (Basoo *et al*., 2012; Khajali *et al*., 2014).

Arginine has distinct roles in stimulation of growth. The growth-promoting polyamines including putrescine, spermidine and spermine are produced from

ARG (Khajali and Wideman, 2010). Results with respect to the effect of ARG on gut cell proliferation (absorptive villi in small intestine) are conflicting. While some results showed no effect of ARG on gut function (Khajali *et al*., 2011; Murakami *et al*., 2012), there are some reports indicating a significant impact of ARG on entrocyte proliferation (Khajali *et al*., 2014; Tan *et al*., 2014). The present study aimed at supplementing ARG to reduced-protein diets to investigate whether this nutritional approach is able to modulate the development of PHS and enhance gut function in broiler chickens reared at high altitude.

Materials and Methods

Birds and experimental facility

The experiment was carried out in the experimental facility of Shahrekord University, Iran, with an altitude of 2,100 m above sea level. A total of 156 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. All chicks were kept, maintained and treated according to the accepted standards for the human treatment of animals. Day-old broiler chicks were raised on a commercial diet until five days of age. Five-day-old chicks were then allocated to pens (13 birds per pen with weighted 89 g on average) so that all pens had equal initial body weights (1157 ± 10 g). The house temperature was set at 32 ± 1°C during Week 1, 25 ± 1°C for Week 2, 20 ± 1°C for Week 3, and 15 ± 1°C until the end of trial (42 days of age) as previously described (Khajali and Saedi, 2011). Birds were subjected to 23 hrs light and 1 hr dark throughout the trial. Birds had free access to feed and water.

Treatments

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 reduced-protein diet (designated as RPD) was prepared with 30 g/Kg less crude protein compared to the level in NPD. The RPD diet was also supplemented with 4 g/Kg L-arginine (Evonik Degussa, Tehran, Iran) and designated as RPD + ARG.

All three diets had the same level of metabolizable energy and offered in mash feed. Potassium carbonate was also added to the reduced-protein diets to equalize the dietary electrolyte balance (Na+K-Cl) between all dietary treatments. The composition of diets are shown in Tables 1. Before the onset of experiment, feed ingredients and mixed diets were sampled and analyzed for crude protein and amino acid contents. Duplicate samples of each ingredient or diet were hydrolyzed in 6 *N* HCl for 24 hrs at 110°C (Andrews and Baldar, 1985).

Upon acid hydrolysis, dietary samples were analyzed for amino acid content by an ion-exchange chromatograph (LKB Biochrom 4141; Cambridge, UK).

Table 1: Composition of normal- and reduced-protein diets fed to broilers in the starter and grower periods

	Starter (5-21 d)		Grower (21-42 d)	
Ingredients (g/Kg unless noted)	NPD[1]	(RPD[2]) & (RPD + ARG[3])	NPD[1]	(RPD[2]) & (RPD + ARG[3])
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	15.5	15	13	14
Oyster shell	14.0	14.5	15.0	15.5
Sodium chloride	3.5	3.5	3.0	3.0
DL-Methionine	1	2	1	2
L-Lysine HCl	-	-	-	2
Vitamin premix[4]	2.5	2.5	2.5	2.5
Trace mineral premix[5]	2.5	2.5	2.5	2.5
Potassium carbonate	-	2	-	2.5
Diet composition				
Analyzed crude protein (g/Kg)	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 ME (Kcal/Kg)	3200	3200	3200	3200
Calculated Na+ K - Cl (meq/Kg)	235	236	222	222

[1]Normal protein diet; [2]Reduced-protein diet; [3]Reduced-protein diet plus L-arginin which prepared by adding 4 g/Kg L-arginin to reduced-protein diet.

[4]Provided the following per Kg of diet: vitamin A (trans-retinyl acetate), 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.

[5]Provided the following per Kg of diet: Mn (from MnSO4·H2O), 40 mg; Zn (from ZnO), 40mg; 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.

Measurements

Records of daily feed intake and weight gain were obtained during 5-42 d period. Feed conversion ratio was also calculated and corrected for mortality body weights. At 42 days of age, 8 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 min to obtain sera. Serum samples were used for the determination

of NO. Serum NO (nitrate + nitrite) was measured according to Behrooj *et al.* (2012).

Quantitative real-time PCR Analysis

At 42 days of age, 8 chickens from the NPD, RPD, and RPD + ARG were randomly selected, weighed and killed by decapitation. The hearts and the lungs were harvested and immediately frozen in liquid nitrogen and stored at -70 ^{o}C for subsequent RNA analysis. Total RNA from the tissues was extracted using RNX-Plus reagent (Sinaclon Bioscience, Tehran, Iran). Homogenized tissue (100 mg) was prepared in digestion buffer. The homogenate was mixed with chloroform. After centrifuging the mixture, total RNA settled in the upper aqueous phase. Following precipitation with isopropanol, the RNA pellet was rinsed with 75% ethanol. The samples of RNA were resuspended in DEPC-treated water. To remove eventual residual DNA, the RNA was treated by DNase (Sinaclon Bioscience, Tehran, Iran); the RNA was then measured and qualified by spectrophotometry. Only RNA with an absorbance ratio (A260/A280) of >1.9 was used for synthesis of cDNA. Total RNA was reverse transcribed into cDNA using PrimeScript™ RT Reagent Kit (Takara Bio Inc., Japan). The reverse transcription mix was heated to 85°C for 5 s to inactivate reverse transcriptase and denature the RNA and then stored at -20°C.

The levels of iNOS (inducible nitric oxide synthase), ET-1 and β-actin transcripts were determined by real-time PCR using SYBR® Premix Ex Taq™ II (Takara Bio Inc., Japan). In order to normalize the input load of cDNA among samples, β-actin was used as an endogenous standard. Details of the specific primer pairs were described else where (Ahmadipour *et al.*, 2015) and listed in Table 2. PCRs were done in a real-time thermo cycler (Rotor Gene Q 6000, Qiagen, USA) in three replicates for each sample of ventricles. One microliter cDNA was added to the 10 µl of SYBR® Premix Ex Taq II Mix and 0.5 µM of each specific primer in a total volume of 20 µl. The thermal profile was 95°C for 30 s, 40 cycles of 94°C for 40 s, 64°C for 35 s and 72°C for 30 s. At the end of each phase, the measurement of fluorescence was done and used for quantitative objectives. Gene expression data were normalized to β-actin. Data were analyzed using Rotor Gene-software, version 2.0.2 (build 4) (Qiagen, Hilden, Germany) and LinRegPCR software version 2012.0 (Amsterdam, Netherland), to give the threshold cycle number and reaction efficiency (Ruijter *et al.*, 2009). Relative transcript levels were calculated using efficiency adjusted Pfaffl methodology (Dorak, 2006).

Table 2: Primers used for quantitative real-time PCR analysis of chicken mRNAs

Target	Primers	PCR Product	Accession No.
β-Actin	5'-AGCGAACGCCCCCAAAGTTCT-3' 5'-AGCTGGGCTGTTGCCTTCACA-3'	139 bp	NM-205518.1
iNOS[1]	5'-AGGCCAAACATCCTGGAGGTC-3' 5'-TCATAGAGACGCTGCTGCCAG-3'	371 bp	U46 504
ET-1[2]	5'-GGACGAGGAGTGCGTGTATT-3' 5'-GCTCCAGCAAGCATCTCTG-3'	141 bp	XM418943

[1]inducible nitric oxide synthase; [2]Endothelin-1.

Assessment of intestinal morphology

At the same day (Day 42), 8 additional birds per treatment were euthanized for intestinal morphology. The intestinal morphology variables, i.e., villus sizes (length, width, crypt depth, and absorptive surface area) were evaluated in the duodenum and jejunum. From each treatment 2-cm segments of the midpoint of the duodenum and the midpoint between the bile duct entry and Meckel's diverticulum (jejunum) were dissected. The segments were flushed with phosphate buffered saline (PBS, pH=7), fixed in Clark fixative for 45 min, and then left in ethyl alcohol for a longer storage. Intestinal segments were subjected to periodic acid-Schiff reagent for 2–3 min for staining. Muscle layer was removed from mucosa, and rows of villi were cut in sagittal sections. The cuts were positioned on glass slides and covered with a coverslip. These samples were evaluated with an optical microscope. Morphometric criteria including villus length, villus width, crypt depth were assessed and then absorptive surface area calculated using the formula = (π) × (VW) × (VL); VW is villus width and VL is villus length. Villus length was measured from the top of the villus to the top of the lamina propria.Villus width was averaged from one-third and two-third of each villus. Crypt depth was measured as the distance from the base of the villus to the sub mucosa (Hassanpour *et al*., 2013).

Assessment of right ventricular hypertrophy

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 PHS was checked daily and whenever the RV:TV was greater than 0.25 were considered as developing pulmonary hypertension (Khajali *et al*., 2014).

Electrocardiographic Recording

Eight chicks per treatment were randomly selected at day 40 and electrocardiograms (ECG) were recorded by an automatic instrument (Kenz ECG 110, Suzuken Japan. Co., Ltd) while standardized at 10 mm = 1 mV with a chart

speed of 50 mm/s. 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. In cases of sampling within pens, data were subjected to a nested design. The statistical model used for growth performance data was $Y_{ij} = \mu + T_i + e_{ij}$. For sera (to measure NO) and carcass (to evaluate gene expression, heart percentage, and RV:TV) data, 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. Any data requiring log transformation were back transformed for presentation of data. Means were separated by the Duncan's multiple range test.

Results

The effects of feeding NPD, RPD and RPD + ARG on growth performance, cardiac/electrocardiographic (lead II) variables and serum nitric oxide of broilers are presented in Table 3. The daily body weight gain was not different between the treated groups of chickens. Feed intake of chickens was decreased in RPD + ARG treatment compared to NPD (P=0.048). Feed conversion ratio of chickens fed RPD +ARG was improved compared to RPD group (P=0.001). The proportion of heart to live body weight (P=0.003) and RV:TV (P=0.021) were increased in chickens fed RPD compared to other groups. Serum NO concentration was significantly higher (P=0.020) in the chickens fed NPD and RPD + ARG when compared with the chickens fed RPD.

Table 3: Performance, cardiac/electrocardiographic (lead II) variables and serum nitric oxide of broiler chickens fed diets with different protein contents and arginine levels

Variables	NPD[1]	RPD[2]	RPD + ARG[3]	*SEM*	*P-value*
daily weight gain (g/bird)	63.5	60.4	61.0	1.17	0.188
daily feed intake (g/bird)	119.3[a]	114.6[ab]	110.7[b]	2.13	0.048
Feed conversion ratio	1.87[a]	1.90[a]	1.81[b]	0.01	0.001
Heart/live body weight (%)	0.58[b]	0.72[a]	0.66[a]	0.026	0.003
RV:TV	0.24[b]	0.30[a]	0.22[b]	0.018	0.021
Serum NO (µM)	15.60[a]	9.91[b]	15.76[a]	1.54	0.020
R wave (mV)	0.21	0.22	0.23	0.033	0.862
S wave (mV)	-0.30[b]	-0.38[a]	-0.29[b]	0.023	0.020
T wave (mV)	0.15	0.18	0.14	0.026	0.455
Number of chickens	8	8	8	-	

[1]Normal protein diet; [2]Reduced-protein diet; [3]Reduced-protein diet plus L-arginin which prepared by adding 4 g/Kg L-arginin to reduced-protein diet.

[a,b]Means in the same row with different letters are significantly different ($P<0.05$).

Real-time PCR results of ET-1 and iNOS genes are shown in Table 4. The relative gene expression of ET-1 in both heart (P=0.004) (right ventricle) and lung (P=0.033) of chickens fed RPD was higher (P=0.020) than chickens fed NPD and RPD + ARG. The relative gene expression of iNOS in the heart (right ventricle) and lung of chickens was not significant between experimental treatments.

Table 4: Relative expression of genes in the heart and lung of broiler chickens fed diets with different protein contents and arginine levels

Variables	NPD[1]	RPD[2]	RPD + ARG[3]	*SEM*	*P-value*
Heart (right ventricle)					
iNOS[4]	0.019	0.017	0.019	0.003	0.455
ET-1[5]	0.138[b]	0.379[a]	0.101[b]	0.063	0.004
Lung					
iNOS[4]	0.142	0.206	0.133	0.053	0.177
ET-1[5]	0.339[b]	0.524[a]	0.332[b]	0.058	0.033
Number of chickens	8	8	8	-	

[1]Normal protein diet; [2]Reduced-protein diet; [3]Reduced-protein diet plus L-arginin which prepared by adding 4 g/Kg L-arginin to reduced-protein diet; [4]inducible nitric oxide synthase; [5]Endothelin-1.
[a,b]Means in the same row with different letters are significantly different ($P<0.05$).

Morphometric measurements of the small intestine in two parts (duodenum and jejunum) are presented in Table 5. Villus height, villus width, crypt depth and absorptive surface area were not significant between the experimental treatments.
Electrocardiographic wave amplitudes (R, S and T waves) of chickens were depicted in Table 3. S wave amplitude was greater (P=0.020) in RPD group than NPD and RPD + ARG groups. Variations of R and T wave amplitudes were not significant among the treatments.

Table 5: Intestinal morphology of broiler chickens fed by diets with different protein contents and arginine levels

Variables	NPD[1]	RPD[2]	RPD + ARG[3]	*SEM*	*P-value*
Duodenum:					
Villus height	1.49	1.42	1.45	0.049	0.534
Villus width	0.57	0.47	0.48	0.044	0.210
Crypt depth	0.47	0.46	0.45	0.015	0.357
Surface area (mm^2)	2.75	2.11	2.16	0.255	0.177
Jejunum:					
Villus height	1.21	1.15	1.17	0.034	0.510
Villus width	0.44	0.41	0.44	0.022	0.471
Crypt depth	0.33	0.34	0.32	0.011	0.818
Surface area (mm^2)	1.67	1.48	1.62	0.112	0.474
Number of chickens	8	8	8	-	

[1]Normal protein diet; [2]Reduced-protein diet; [3]Reduced-protein diet plus L-arginin which prepared by adding 4 g/Kg L-arginin to reduced-protein diet.
No significant difference was observed between treatments in each variable ($P>0.05$).

Discussion

This study evaluated the modulating effect of L-arginine on the development of PHS in broilers fed on reduced-protein diets. There was no significant change in daily weight gain of broilers in all of treatments. In agreement with our finding, Saki *et al.* (2013) and Khajali *et al.* (2014) reported that dietary L-arginine supplementation had no significant effect on weight gain of broilers raised under hypobaric conditions. However, feed conversion ratio was improved in chickens receiving reduced-protein diets plus L-arginine (4 g/Kg). L-arginine supplementation could influence feed conversion efficiency in several ways such as positive effect on the protein/DNA synthesis, cell proliferation (Khajali and Wideman, 2010) and also regulation of intermediate metabolism by modifying hepatic lipogenesis (Sharifi *et al*., 2015).

The proportion of heart weight and RV:TV ratio as well as S wave amplitude of electrocardiogram were increased as dietary protein declined but such increases were modulated by L-arginine supplementation. Increased relative weight of heart reflects overload of pumping activity for greater oxygenation (Izadinia *et al*., 2010). An increase in the RV:TV ratio and S wave amplitude reflects the hypertrophy of the right ventricular wall that can be directly related to increased pulmonary arterial pressure (Hassanpour *et al*., 2009b; Yousefi *et al*., 2013). Elevation of the pulmonary arterial pressure theoretically can be attributed to increase in cardiac output as well as in pulmonary vasculature resistance. Wideman *et al*. (2001) confirmed that RV:TV ratio could be as an critical index to indicate the extremity of pulmonary hypertension in the broiler chickens. Therefore, on the basis of this index, the present study determined that the reduced-protein diet could develop pulmonary hypertension, which is in agreement with Behrooj *et al.* (2012). On the other hand, L-arginine supplement could inhibit developing of this syndrome. L-arginine is the NO precursor that acting as the key pulmonary vasodilator and modulator of vasoconstriction. Adding supplemental L-arginine to broiler diets facilitated pulmonary vasodilation in response to large increases in blood flow (Wideman *et al*., 1996) and modulated the pulmonary hypertension (Sharifi *et al.,* 2015). The responses to increases in the pulmonary arterial pressure include cardiac work hypertrophy that is specific for the right ventricle (elevated RV:TV ratio), and accelerated rates of blood flow through the lungs (Balog, 2003). In the present study, adding L-arginine to reduced-protein diet considerably increased serum nitric oxide which might be the reason for reduced the RV:TV ratio and consequently reduced pulmonary hypertension. Though this elevation of nitric oxide was not seemingly due to up-regulation in iNOS gene expression. Many studies also showed that inclusion of L-arginine in broiler diets improved nitric oxide concentration in plasma with a concomitant reduction in right ventricle to total ventricle weight ratio (Tan *et al*., 2005; Basoo *et al*., 2012; Khajali *et al*., 2014).

Our data showed a down-regulation of ET-1 gene expression in the heart and lung of chickens fed reduced-protein diet supplemented with L-arginine. ET-1 is the most potent vasoconstrictor substance produced by the cardiovascular system. The pathophysiological role of ET-1 has been proposed in PHS (Shah, 2007). It has

been confirmed that high altitude-induced PHS is associated with increased gene expression of ET-1 in the heart of chickens (Ahmadipour *et al*., 2015). Accordingly, down-regulation of this gene may decelerate development of hypobaric pulmonary hypertension as reported in chickens fed reduced-protein diet supplemented with L-arginine. Regulation of ET-1 is multifactorial. Interactions between ET system and several factors including hypoxia, nitric oxide, angiotensin II, catecholamines, cytokines and growth factors determine gene expression and circulatory level of ET-1. Apparently, adding arginine to diet of chickens would divert these interactions (via NO production) to reduce gene expression of ET-1 (Shah, 2007; Alonso and Radomski, 2003).

Our study showed that reducing protein in diet had not harmful effect on the intestinal morphology in broilers reared at high altitude, and supplementation of L-arginine at 4 g/Kg did not affect intestinal status. However, Khajali *et al*. (2014) reported that higher levels of arginine supplement (10 g/Kg) could improve intestinal morphology in chickens.

In conclusion, supplemental L-arginine in reduced protein diets played complementary roles to increase nitric oxide bioavailability and decrease ET-1 gene expression, thus improving cardio-pulmonary performance and prevent the development of PHS in broiler chickens.

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References

Ahmadipour B, Hassanpour H, Asadi E, Khajali F, Rafiei F & Khajali F. 2015. *Kelussia odoratissima* Mozzaf-A promising medicinal herb to prevent pulmonary hypertension in broiler chickens reared at high altitude. Journal of Ethnopharmacology, 159: 49-54.

Aletor VA, Hamid II, Niess E & Pfeffer E. 2000. Low-protein amino acid-supplemented diets in broiler chickens: effects on performance, carcass characteristics, whole-body composition and efficiencies of nutrient utilisation. Journal of the Science of Food and Agriculture, 80: 547-554.

Alonso D & Radomski MW. 2003. The nitric oxide-endothelin-1 connection. Heart Failure Reviews, 8: 107-115.

Andrews RP & Baldar NA. 1985. Amino acid analysis of feed constituents. Science Tools, 32: 44–48.

Balog JM. 2003. Ascites syndrome (pulmonary hypertension syndrome) in broiler chickens: are we seeing the light at the end of the tunnel? Avian and Poultry Biology Reviews, 14: 99-126.

Basoo H, Khajali F, Asadi Khoshoui E, Faraji M & Wideman RF. 2012. Re-evaluation of arginine requirements for broilers exposed to hypobaric condition during the 3-to 6-week period. Journal of Poultry Science, 49: 303-307.

Behrooj N, Khajali F & Hassanpour H. 2012. Feeding reduced-protein diets to broilers subjected to hypobaric hypoxia is associated with the development of pulmonary hypertension syndrome. British Poultry Science, 53: 658-664.

Dorak MT. 2006. Real Time PCR. 1st Ed. Taylor and Francis, Oxford Publications. UK. 333 Pages.

Hassanpour H, Yazdani A, Khabir Soreshjani K & Asgharzadeh S. 2009a. Evaluation of endothelial and inducible nitric oxide synthase genes expression in the heart of broiler chickens with experimental pulmonary hypertension. British Poultry Science, 50: 725-732.

Hassanpour H, Moghaddam AKZ & Zarei H. 2009b. Effect of citric acid on electrocardiographic parameters of broiler chickens with pulmonary hypertension. Acta Veterinaria Hungarica, 57: 229-238.

Hassanpour H, Teshfam M, Momtaz H, Brujeni GN & Shahgholian L. 2010. Up-regulation of Endothelin-1 and Endothelin type A receptor genes expression in the heart of broiler chickens versus layer chickens. Research in Veterinary Science, 89: 352-357.

Hassanpour H, Momtaz H, Shahgholian L, Bagheri R, Sarfaraz S & Heydaripoor B. 2011. Gene expression of endothelin-1 and its receptors in the heart of broiler chickens with T 3-induced pulmonary hypertension. Research in Veterinary Science 91: 370-375.

Hassanpour H, Zamani Moghaddam AK, Khosravi M & Mayahi M. 2013. Effects of synbiotic on the intestinal morphology and humoral immune response in broiler chickens. Livestock Science, 153: 116-122.

Hassanzadeh M, Buyse J & Decuypere E. 2008. Further evidence for the involvement of anatomical parameters of the cardiopulmonary system in the development of ascites syndrome in broiler chickens. Acta Veterinaria Hungarica, 56: 71-80.

Izadinia M, Nobakht M, Khajali F, Faraji M, Zamani F, Qujeq D & Karimi I. 2010. Pulmonary hypertension and ascites as affected by dietary protein source in broiler chickens reared in cool temperature at high altitudes. Animal Feed Science and Technology, 155: 194-200.

Kamran Z, Sarwar M, Nisa M, Nadeem MA, Mahmood S, Babar ME & Ahmed S. 2008. Effect of low-protein diets having constant energy-to-protein ratio on performance and carcass characteristics of broiler chickens from one to thirty-five days of age. Poultry Science, 87: 468-474.

Khajali F, Khoshouie EA, Dehkordi SK & Hematian M. 2008. Production performance and egg quality of Hy-line W36 laying hens fed reduced-protein diets at a constant total sulfur amino acid: lysine ratio. Journal of Applied Poultry Research, 17: 390-397.

Khajali F & Wideman RF. 2010. Dietary arginine: metabolic, environmental, immunological and physiological interrelationships. World's Poultry Science Journal, 66: 751-766.

Khajali F & Saedi M. 2011. The effect of low chloride and high bicarbonate diets on growth, blood parameters, and pulmonary hypertensive response in broiler chickens reared at high altitude. Archiv für Geflügelkunde, 75: 235-238.

Khajali F, Tahmasebi M, Hassanpour H, Akbari MR, Qujeq D & Wideman RF. 2011. Effects of supplementation of canola meal-based diets with arginine on

performance, plasma nitric oxide, and carcass characteristics of broiler chickens grown at high altitude. Poultry Science, 90: 2287-2294.

Khajali F, Moghaddam MH & Hassanpour H. 2014. An L-Arginine supplement improves broiler hypertensive response and gut function in broiler chickens reared at high altitude. International Journal of Biometeorology, 58: 1175-1179.

Murakami AE, Fernandes JI, Hernandes L & Santos TC. 2012. Effects of starter diet supplementation with arginine on broiler production performance and on small intestine morphometry. Pesquisa Veterinaria Brasileira, 32: 259-266.

NRC (National Research Council). 1994. Nutrient Requirements of Poultry. 9th Rev. Ed. National Academy Press. Washington, DC. 176 Pages.

Ruijter JM, Ramakers C, Hoogaars WMH, Karlen Y, Bakker O, Van den Hoff MJB & Moorman AFM. 2009. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Research, 37: 1-12

Ruiz-Feria CA. 2009. Concurrent supplementation of arginine, vitamin E, and vitamin C improve cardiopulmonary performance in broilers chickens. Poultry Science, 88: 526-535.

Saki A, Haghighat M & Khajali F. 2013. Supplemental arginine administered *in ovo* or in the feed reduces the susceptibility of broilers to pulmonary hypertension syndrome. British Poultry Science 54: 575-580.

SAS Institute Inc. 2007. *SAS/STAT User's Guide Release 9*. SAS Institute Inc., Cary, NC.

Shah R. 2007. Endothelins in health and disease. European Journal of Internal Medicine, 18: 272-282.

Sharifi M, Khajali F, Hassanpour H, Pour-Reza J, & Pirany N. 2015. L-Arginine supplementation of reduced-protein diets improves pulmonary hypertensive response in broiler chickens reared at high altitude. British Poultry Science, DOI:10.1080/00071668.2015.1054258 (published online).

Tan X, Pan JQ, Li JC, Liu YJ, Sun WD & Wang XL. 2005. L-Arginine inhibiting pulmonary vascular remodelling is associated with promotion of apoptosis in pulmonary arterioles smooth muscle cells in broilers. Research in Veterinary Science, 79: 203-209.

Tan X, Hu SH & Wang XL. 2007. Possible role of nitric oxide in the pathogenesis of pulmonary hypertension in broilers: a synopsis. Avian Pathology, 36: 261-267.

Tan J, Applegate TJ, Liu S, Guo Y & Eicher SD. 2014. Supplemental dietary L-arginine attenuates intestinal mucosal disruption during a coccidial vaccine challenge in broiler chickens. British Journal of Nutrition, 7: 1098-1109

Wideman RF. 2001. Pathophysiology of heart/lung disorders: pulmonary hypertension syndrome in broiler chickens. World's Poultry Science Journal, 57: 289-307.

Wideman RF, Kirby YK, Tackett CD, Marson NE & McNew RW. 1996. Cardio-pulmonary function during acute unilateral occlusion of the pulmonary artery in broilers fed diets containing normal or high levels of arginine-HCl. Poultry Science, 75: 1587-1602.

Yousefi A, Khajali F, Hassanpour H & Khajali Z. 2013. Dietary L-carnitine improves pulmonary hypertensive response in broiler chickens subjected to hypobaric hypoxia. Journal of Poultry Science, 50: 143-149.