

EFFECTS OF ARGININE ON CYTOKINES AND NITRIC OXIDE SYNTHESIS IN BROILERS

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ABSTRACT

The effect of dietary supplement of arginine on immunoglobulin, cytokine and nitric oxide (NO) synthesis in broilers was investigated. Three hundred one-day-old Arbor Acre mixed-sex broiler chickens were randomly allotted to five dietary treatments with six replicates in each treatment and ten chickens within each replicate. The five treatments viz., the basal diet with 0 (arginine-deficient group), 4.5 (arginine-normal group, control), 9.0, 13.5, and 18.0 g/kg arginine. The feeding experiment period was 42 days. The results of *in vivo* experiments showed that with increasing levels of dietary arginine, interleukin (IL)-2, NO content and inducible nitric oxide synthase (iNOS) activity in serum as well as iNOS mRNA expression in the ileum and liver of broiler chickens increased quadratically at the age of 21 d ($P < 0.05$). In addition, positive effects of arginine tended to be suppressed when addition of arginine in the diet was increased to 18.0 g/kg. The results of *in vitro* experiment indicated that, IL-2 and iNOS content increased quadratically with increasing addition of arginine ($P < 0.01$), and 80 $\mu\text{g/mL}$ arginine supplement group had the highest value. Dietary arginine improved the levels of IL-2, NO and iNOS expression in a quadratic dose-dependent manner.

Key words: Arginine, Broiler, Cytokine, Nitric Oxide Synthase, mRNA Expression.

INTRODUCTION

The L-arginine, one of the 20 most common natural amino acids (AA), is classified as a semi-essential or conditionally essential AA for mammals and essential AA for birds due to their inability to synthesize arginine (Khajali and Wideman, 2010).

It has been reported that arginine could regulate the immune function via nitric oxide (NO) pathway (Amber *et al.*, 1991; Tsai *et al.*, 2002). Arginine in animals has two chief direct metabolic pathways: ① Arginine is decomposed into ornithine and urea by arginase; ② Arginine and molecular oxygen generate citrulline and NO by nitric oxide synthase (NOS). NO is produced in different cells of the immune system (macrophage, dendritic cells, NK cells, mast cells and phagocytic cells *etc.*) and other cells involved in immune reactions (endothelial cells, epithelial cells and hepatocyte *etc.*). NO, as a key signal molecule, plays a pivotal role in modulating immune response and inflammation through multiple pathways (Moilanen *et al.*, 1999; Korhonen *et al.*, 2005; Jobgen *et al.*, 2006). NO can enhance the body's non-specific immunity by non-specifically killing pathogens such as bacteria, fungi, parasites and tumor cells. More importantly, NO regulates the synthesis and secretion of some immune cytokines, such as tumor necrosis factor (TNF), prostaglandin E₂ (PGE₂), interleukin (IL) and interferon (IFN), which leads to a more wide influence on the immune function. Inducible nitric oxide synthase (iNOS), one of three known forms of NOS, is controlled at the

transcriptional level and is important in modulating immune responses. Macrophages and myoblasts in chickens can express iNOS (Lin *et al.*, 1996; Shimizu *et al.*, 1998; Li *et al.*, 2009).

Previous research showed that arginine in diet was not high enough to support the highest immunity in animals (Wideman *et al.*, 1996; Ruiz-Feria *et al.*, 2001). Kazuaki (1999) reported that deficiency of dietary arginine appears to limit the maximum amount of NO produced. Wu and Meininger (2002) demonstrated that inadequate intake of dietary arginine impairs NO synthesis by both constitutive and inducible NOS in animals, indicating a role of adequate arginine nutrition in immune function. Moreover, Bulgrinet *al.* (1993) and Peck *et al.* (1995) indicated that an increase in dietary arginine level correlated positively with NO production in animals.

The present study was conducted to investigate the effects of different levels of arginine supplement in diets on cytokine, NO content, iNOS activity, and expression of iNOS mRNA *in vitro* and *in vivo* in order to explore the possible mechanisms of arginine affecting the immune function in broiler chickens.

MATERIALS AND METHODS

The experimental protocol was approved by Animal Care and Use Committee of Inner Mongolia Agricultural University, Huhhot, China.

Experimental design

Animal experiments: A total of 300 one-day-old Arbor Acre mixed-sex broiler chickens were randomly allotted to five dietary treatments with six replicates in each treatment and ten chickens in each replicate. There were no significant differences in initial body weight among treatments ($P > 0.05$). The five treatments consisted of basal diets supplemented with either 0 (Arginine-deficient group), or 4.5 (Arginine-normal group, Control), 9.0, 13.5 and 18.0 g/kg L-arginine. The basal diet met the nutrient requirements suggested by NRC (NRC, 1994) except arginine, and the control diet contained normal levels of arginine (Table 1). Arginine was provided by the Beijing Jiakangyuan Biotechnology Co., Ltd. The trial lasted six weeks and was divided into two periods (d 1 to 21 and d 22 to 42). Diets and water were supplied *ad libitum* during the whole experimental period. A photoperiod of 23-h light/ 1-h dark was maintained. Room temperature was set at approximately 32°C in the first week, and then reduced by 3°C weekly until reaching 20°C, which was then maintained until the end of the experiment.

On days 21 and 42, one bird from each replicate of every treatment was randomly selected for blood collection via wing veins in a 5-mL evacuated tube. The blood samples were subsequently centrifuged at 1,200 ×g for 10 min at 4°C to yield serum and later stored at -20°C until analysed for cytokine, NO and iNOS. The birds were then killed and the small intestinal, liver and spleen were quickly removed and frozen in liquid nitrogen, and then stored at -80°C. These were used to determine relative expression of iNOS mRNA.

In vitro experiments: Briefly, 3 mL blood was collected from the wing veins of a 21-day-old broiler in arginine-normal group, and added slowly into a 10-mL tube which contained 4 mL of lymphocyte-stratified fluid (Tianjin Blood Research Center, China). The tube was centrifuged at 800×g for 30 min. Lymphocytes in the middle layer were removed to another tube, and were washed 3 times by Hank's fluid with free Ca²⁺ and Mg²⁺. The lymphocytes were then suspended in RPMI-1640 (Gibco, UK) complete medium supplemented with 10% (v/v) of fetal calf serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and 25 mmol/L of N-(2-hydroxyethyl)-piperazine-N'-2-ethane-sulphonic acid. Number of live cells were counted and adjusted to the density of 2 × 10⁶ cells per mL.

Test culture system was 500 μL, including 487.5 μL lymphocyte suspension and 12.5 μL solution of arginine with different concentrations. Arginine concentrations were five treatments (0, 40, 80, 160 and 320 μg/mL) with eight replicates in each treatment. The plates were placed in an incubator and cultured at 37°C with 5% CO₂ for 24 h. The Concanavalin A (Con A; Sigma, USA; final concentration 16 μg/mL) was added

into each well. The plates continued to be cultured for 24 h. Finally, the supernatant was collected in a sterile laminar flow hood, and stored at -20°C until analysed for cytokine, NO and iNOS. Lymphocytes were washed 3 times with PBS, and used to determine relative expression of iNOS mRNA.

Sample analysis: Cytokine contents were assayed by using commercially available kits (Beijing Sino-uk institute of biological technology). NO content and iNOS activity were assayed using commercially available NO kits and iNOS kits (Nanjing Jiancheng Institute of Bioengineering), according to the manufacturer's instructions.

Total RNA isolation and reverse transcription (RT):

All steps were carried out under RNase-free conditions. Total RNA was isolated from the ileum, liver and spleen using the RNAiso Reagent (TaKaRa Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's directions. The RNA purity was gauged using UV-clear Microplates (BioTek) at OD260 and an OD260/OD280 ratio. The OD260/OD280 ratio of all samples was above 1.80 and below 2.2. RNA completeness was tested electrophoretically by ethidium bromide staining. Total RNA was reverse-transcribed using the PrimeScript[®] RT Master Mix Perfect Real Time Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China) following the manufacturer's instructions. This process consists of reverse transcription (37°C, 15 min) and reverse transcriptase inactivation (85°C, 5 sec). The RT reaction mixture (10 μL) contained 2 μL 5×PrimeScript[®] RT Master Mix (for Real Time), total RNA containing 500 ng base and RNase free dH₂O adding up to 10 μL. The RT products (cDNA) were stored at -20°C pending quantitative PCR assay.

Real-time PCR for quantification of iNOS mRNA:

Relative levels of iNOS mRNA were quantified using SYBR[®] Premix Ex Taq[™] II (ThiRNaseH Plus) Kit (TaKaRa Biotechnology Co., Ltd. Dalian, China) according to manufacturer's instructions and a light cycler real-time DNA amplification system (MJ research Opticon-2, USA) following optimized PCR protocols. Reactions were also performed with negative controls (water replacing cDNA). Primers used were as follows: -Actin (118bp, GenBank accession no. NM_205518) 5'-GCCAACAGA GAGAAGATGACAC-3' (forward) and 5'-GTAACACCAT CACCAGAGTCCA-3' (reverse); iNOS (371bp, GenBank accession no. U46504) 5'-AGGCCAAACATCCTGGAGGTC-3' (forward) and 5'-TCATAGAGACGCTGCTGCCAG-3' (reverse). The PCR reaction system (20 μL) was performed in 10.0 μL 2×SYBR[®] Premix Ex Taq[™] II and 0.4 μL (10 μM) each of forward and reverse specific primers, 2 μL of cDNA template and 7.2 μL RNA Free H₂O. The same dilution was used for both iNOS and -actin.

The following experimental PCR reaction was done at 95°C for 1 min, 5 sec at 95°C for denaturation, 30 sec at 62°C for annealing, and 10 sec at 72°C for extension. Test of the fluorescent product was fulfilled at the end of melting curve program (70 to 95°C with a heating rate of 0.5°C/s and a continuous fluorescence measurement). Fluorescence data was acquired after the extension step during PCR reactions that contained SYBR Green I. The iNOS and β -Actin gene amplification of the PCR products were confirmed by gel electrophoresis on 1.0% agarose stained with ethidium bromide, and bands were visualized by exposure to ultraviolet light. Sequences were confirmed by Sangon Biological Engineering Technology and Services Co., Ltd (Shanghai, China). Thereafter, PCR products were analyzed by generating a melting curve. The melting curve of a product is sequence-specific and can be used to differentiate non-specific from specific PCR products. Amplification of a dilution series of iNOS mRNA and β -actin mRNA shaped the amplification efficiency curves. The melting curve after amplification showed a single melting peak, indicating a specific product. Relative expression levels of iNOS were calculated using the 2^{-Ct} method, where $Ct = Ct \text{ iNOS} - Ct \text{ } \beta\text{-actin}$ (Schmittgen *et al.*, 2000).

Statistical analysis: All data were subjected to General Linear Model procedure of SAS software (SAS Version 9.0, SAS Institute, 2003) to determine the statistical significance of the treatment groups. Regression analysis was conducted to evaluate linear and quadratic effects of arginine on the various response criteria. A level of $P < 0.05$ was used as the criterion for statistical significance.

RESULTS

Cytokine content in serum: The effect of arginine supplementation on the serum interleukin content of broilers has been shown in Table 2. Lack of arginine reduced interleukin content. Broilers fed diets with 9~13.5g/kg arginine had higher interleukin concentrations in serum. On day 21, IL-2 content increased quadratically with increasing addition of arginine ($P = 0.044$), and 9.0 g/kg arginine supplement group had the highest value.

NO content and iNOS activity in serum: Lack of arginine reduced NO content and iNOS activity (Table 3). Broilers fed with arginine-supplemented diets had higher NO concentrations and iNOS activities in serum than those fed with the control diet, and the two indexes increased quadratically with increasing addition of arginine on day 21 ($P = 0.003$, $P = 0.045$), and the 13.5 g/kg arginine supplementation group had the highest value. On day 42, non-significant ($P > 0.05$) linear or quadratic effect was observed for the serum NO content

and iNOS activity in response to increasing supplementation of arginine.

Relative expression of iNOS mRNA: An increase in arginine supplementation resulted in a quadratic ($P = 0.005$, $P = 0.014$) increase in the expression of iNOS mRNA in the ileum and liver on day 21 (Table 4). Compared to the control group, arginine-deficient group and 18.0 g/kg arginine supplement group had low expression of iNOS mRNA in ileum, liver and spleen of broilers. However, arginine supplementation had no effect on the iNOS mRNA expression in ileum and liver on day 42 and in spleen on day 21 and 42. Broilers fed diet supplemented with 9.0 g/kg arginine had the greatest iNOS mRNA expression in ileum, liver and spleen over the entire experimental period. However, supplementation of 4.5 g/kg arginine resulted in the highest iNOS mRNA expression in spleen on day 42. The progressive iNOS mRNA expression reductions occurred when arginine supplement was increased to 13.5 g/kg.

Table 1. Composition and nutritional levels of basal diets (air-dry basis, %)

Ingredients	Content	
	1-21d	22-42d
Corn	62.00	68.46
Corn gluten meal	18.50	19.00
Soybean meal	6.50	0.00
Wheat bran	7.20	7.48
Limestone	1.51	1.41
Dicalcium phosphate	1.90	1.59
Methionine	0.16	0.00
Lysine	0.75	0.60
Threonine	0.05	0.06
Tryptophan	0.06	0.05
Sodium chloride	0.37	0.35
Additive ¹	1.00	1.00
Analysed Nutrient Composition ²		
Metabolic energy (MJ/kg)	12.49	12.81
Crude protein	21.51	19.18
Calcium (Ca)	1.04	0.91
Available phosphorus (AP)	0.46	0.40
Lysine	1.28	0.98
Methionine	0.56	0.38
Arginine	0.86	0.68

¹ Supplemental trace elements and vitamins within a kilogram of basic diet: Iron (Fe) 95 mg; Zinc (Zn) 75 mg; Manganese (Mn) 75 mg; Copper (Cu) 10 mg; Iodine (I) 0.6 mg; Selenium (Se) 0.3 mg; Vitamin A 6,000 IU; Vitamin D 1,250 IU; Vitamin E 15 mg; Vitamin K 2.2 mg; Vitamin B₁ 1.5 mg; Vitamin B₂ 8.0 mg; Vitamin B₆ 2.5 mg; Vitamin B₁₂ 0.011 mg; Niacin 44 mg; D-pantothenic acid 11 mg; Folic acid 0.9 mg; Biotin 0.11 mg; Choline 1100 mg.

² The energy was calculated from the NRC (1994) tabular value.

In vitro experiments: The IL-2 and iNOS content increased quadratically with increasing addition of

arginine ($P=0.005$, $P=0.010$; Table 5), and 80 g/mL arginine supplement group had the highest value. A quadratic increase in NO content and iNOS mRNA

expression was observed with increasing arginine supplementation ($P=0.056$, $P=0.056$).

Table 2. Effects of different levels of dietary arginine on serum interleukin contents in broilers¹

Items	Age	Arginine(g/kg diet)					SEM	p-value	
		0	4.5	9	13.5	18		Linear	Quadratic
IL-1 (ng/mL)	21d	0.27	0.27	0.27	0.27	0.26	0.01	0.915	0.985
	42d	0.29	0.35	0.36	0.39	0.35	0.01	0.091	0.063
IL-2 (ng/mL)	21d	7.41 ^b	8.25 ^{ab}	8.93 ^a	8.20 ^{ab}	7.49 ^b	0.19	0.548	0.044
	42d	9.22	9.28	9.58	9.47	9.19	0.24	0.921	0.906
TNF-a (ng/mL)	21d	1.70	1.73	1.74	1.75	1.74	0.05	0.770	0.941
	42d	0.96	1.11	1.27	1.36	1.28	0.07	0.098	0.170

¹IL= interleukin. TNF = Tumor Necrosis Factor. SEM = Standard error of mean. The number of observations for each mean value was six (n = 6). a,b,c Means within each row without a common superscript differ significantly ($P < 0.05$).

Table 3. Effects of different levels of dietary arginine on serum NO content and iNOS activity in broilers¹

Items	Age	Arginine (g/kg diet)					SEM	p-value	
		0	4.5	9.0	13.5	18.0		Linear	Quadratic
NO ($\mu\text{mol/L}$)	21d	30.14 ^{bc}	33.14 ^{ab}	35.48 ^{ab}	38.56 ^a	24.00 ^c	1.19	0.270	0.003
	42d	21.85	24.49	26.44	25.99	25.99	1.59	0.397	0.600
iNOS (U/mL)	21d	10.79 ^b	17.30 ^{ab}	23.83 ^{ab}	29.63 ^a	18.38 ^{ab}	2.36	0.100	0.045
	42d	16.32	16.31	19.03	16.72	16.49	0.43	0.735	0.486

¹NO = Nitric oxide. iNOS = Inducible nitric oxide synthase. SEM=Standard error of mean. The number of observations for each mean value was six (n = 6). a,b,c Means within each row without a common superscript differ significantly ($P < 0.05$).

Table 4. Effects of different levels of dietary arginine on relative expression of iNOS mRNA in tissues of broilers ($2^{-\Delta\text{Ct}}$)¹

Items	Age	Arginine(g/kg diet)					SEM($\times 10^{-3}$)	p-value	
		0	4.5	9.0	13.5	18.0		Linear	Quadratic
Ileum	21d	0.0036 ^c	0.0053 ^{bc}	0.0076 ^a	0.0050 ^{bc}	0.0035 ^c	0.35	0.890	0.005
	42d	0.0052	0.0067	0.0101	0.0094	0.0064	1.08	0.796	0.506
Liver	21d	0.0030 ^c	0.0051 ^{bc}	0.0071 ^a	0.0052 ^{bc}	0.0034 ^c	0.42	0.825	0.014
	42d	0.0054	0.0071	0.0076	0.0053	0.0051	0.63	0.704	0.690
Spleen	21d	0.0073	0.0087	0.0106	0.0079	0.0078	0.70	0.958	0.643
	42d	0.0065	0.0083	0.0063	0.0060	0.0053	0.63	0.416	0.670

¹iNOS = Inducible nitric oxide synthase. SEM = Standard error of mean. The number of observations for each mean value was six (n = 6). a,b,c Means within each row without a common superscript differ significantly ($P < 0.05$).

Table 5. Effects of different levels of arginine in diets on lymphocyte¹

Items	Arginine (%)					SEM	p-value	
	0	40	80	160	320		Linear	Quadratic
IL-1(ng/mL)	0.06	0.06	0.06	0.06	0.06	0	0.8294	0.8445
IL-2(ng/mL)	1.91 ^b	2.16 ^b	2.80 ^a	2.51 ^{ab}	2.00 ^b	0.09	0.7908	0.0047
TNF- (ng/mL)	0.53	0.58	0.61	0.55	0.44	0.03	0.1093	0.1269
NO($\mu\text{mol/L}$)	4.09	6.21	7.10	5.92	5.01	0.24	0.7805	0.0563
iNOS (U/mL)	8.35	9.99	14.84	11.31	8.39	0.44	0.913	0.0098
Expression of iNOS mRNA ($2^{-\text{Ct}}$)	0.0000	0.0244	0.0185	0.0083	0.0001	0.00	0.0638	0.0563

¹The number of observations for each mean value was six (n = 8). a,b,c Means within each row without a common superscript differ significantly ($P < 0.05$).

DISCUSSION

Cytokine is a class of proteins or small peptides which can pass information between cells and have immunomodulatory functions. Cytokine include interleukin, interferon, and tumor necrosis factor and many more. IL-1 can promote the proliferation of B cells and secrete antibodies. IL-2 is an important broad spectrum enhancement factor in vivo. TNF- can kill or inhibit certain tumor cells in vivo and in vitro. Reynolds *et al.* (1990) and Yeh *et al.* (2002) showed that administration of arginine enhances IL-2 production by lymphocytes. Han *et al.* (2008) reported that adding 0.5% arginine in diets could increase IL-2 concentration in weaned piglets. The present results indicated that arginine supplementation resulted in increased cytokine levels. IL-2 content had a quadratic dose-dependent relationship with arginine added in diets. It has also previously been reported that levels of IL-2 and proliferation of T lymphocyte increase in a dose-dependent manner with dietary arginine (Ochoa *et al.*, 2001; Tayade *et al.*, 2006; Wu *et al.*, 2008).

Bulgrin *et al.* (1993) and Peck *et al.* (1995) indicated that an increase in dietary arginine level correlated positively with NO production in animals. However, deficiency of dietary arginine appears to limit the maximum amount of NO produced. Wu and Meininger (2002) demonstrated that inadequate intake of dietary arginine impaired NO synthesis by both constitutive and inducible NOS in animals, indicating a role for adequate arginine nutrition in immune function. The results of present study are consistent with the results of these studies. Kazuaki's (1999) experiment clearly showed that NO produced by iNOS was also dependent on arginine intake in chicks. Luo *et al.* (2006) showed that adding arginine promoted NOS activity and increased the body's levels of NO in heat-stress mice.

The information related to the effects of arginine on expression of iNOS in poultry are scanty. Hui *et al.* (2011) reported that the short-term application of L-arginine could promote the expression of iNOS in colorectal tumor tissue and elevate serum concentration of NO in patients. In our study, adding arginine improved iNOS activity and enhanced iNOS expression. In addition, a higher level of dietary arginine tended to decrease NO content and iNOS activity and lessen iNOS expression, which suggested that there might be a threshold level of arginine inclusion beyond which progressive iNOS expression was suppressed. However, further work is needed to replicate present findings and to identify the threshold level in broiler chickens.

In conclusion, a quadratic dose-dependent relationship existed between supplemented dosage of arginine and immune indexes such as IL-2, NO content and iNOS activity in serum as well as iNOS mRNA expression in ileum and liver of broiler chickens.

Supplementing arginine resulted in better enhancing effects of what: This implies that the mechanism by which arginine modulates immune functions in chickens may be that arginine inactivates IL-2, NO secretion and iNOS expression.

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