L-Arginine Regulates Immune Functions in Chickens Immunized with Intermediate Strain of Infectious Bursal Disease Vaccine

Jianzhuang Tan1, Yuming Guo1, Todd. J. Applegate2, Encun Du1 and Xu Zhao1

1 State Key Laboratory of Animal Nutrition, College of Animal Science and Technology, China Agricultural University, Beijing 100193, P. R. China
2 Department of Animal Sciences, Purdue University, West Lafayette, IN 47907-2054, USA

The present study investigated the effects of dietary arginine (Arg) supplementation on immune functions of broiler chickens inoculated with infectious bursal disease vaccine (IBDV). A total of 500 one-day-old female Ross (308) broilers were randomly assigned into 10 treatments (5 replicates per treatment, 10 birds per replicate). On day 14, birds were inoculated intramuscularly with IBDV or saline. Birds were fed diets containing one of five dietary Arg concentrations: 9.9, 13.9, 17.6, 21.3, or 25.3g/kg respectively. The IBDV inoculation significantly reduced (P <0.05) serum lysozyme and IgA concentration, mitogen-stimulated peripheral blood mononuclear cell (PBMC) proliferation (Con A), PBMC in vitro NO and H2O2 yield, and serum anti-Newcastle disease virus (NDV) body titers. Increasing Arg concentration linearly increased (P<0.05) serum lysozyme concentration, quadratically increased (P<0.05) in vitro PBMC NO yield, linearly and quadratically increased (P<0.05) PBMC proliferation (LPS). Serum anti-IBDV antibody titers tended to be increased quadratically (P=0.06) by increasing Arg concentration. The Arg requirement of IBDV inoculated chickens (18.9±0.5) for minimum FCR was higher (P<0.05) than that of un-inoculated chickens (16.0±1.3). The Arg requirements of IBDV inoculated chickens for the highest IgA concentration (17.5±0.6g/kg) and PBMC proliferation (LPS) (19.8±2.1g/kg) tended to be higher (P<0.010) than those for un-inoculated chickens (IgA: 16.1±0.6g/kg; PBMC proliferation (LPS): 16.3±0.8g/kg). These results indicate that dietary Arg supplementation may have a potential effect in alleviating IBDV-inoculation induced immunosuppression via enhancing the immune function of chickens.

Key words: arginine, broiler, immunosuppression, infectious bursal disease

Introduction

Infectious bursal disease (IBD) is an acute, highly infectious and immunosuppressive disease caused by IBD virus. The primary target of this virus is the B lymphocytes in the bursa of young chickens (Van Den Berg, 2000), which results in the lysis of B cells and humoral immunosuppression. IBD is a considerable threat to the poultry industry, as it can result in mortality, immunosuppression that renders chickens susceptible to secondary infections, suboptimal response to vaccination, and a great deal of economic loss (Long et al., 2011; Tan et al., 2015). Nutritional strategies have been considered as an effective way to enhance immune function and alleviate immunosuppression in IBD virus infected chickens (Long et al., 2011; Rajput et al., 2010).

Birds are unable to synthesize Arginine (Arg) because they have an incomplete urea cycle, thus, Arg is an essential amino acid for birds. Meanwhile, Arg is a conditional essential amino acid for mammals under pathological status, such as sepsis and trauma, in which animals deplete more Arg than normal requirement for immune response (Luiking et al., 2005; Stechmiller et al., 2005). It has been reported that in some pathologic status, such as coccidial infection, chickens may deplete more Arg than uninfected chickens to produce NO which plays a critical role in parasite killing via direct or indirect (peroxynitrite, ONOO-) actions (Bogdan, 2001), and results in pathological Arg deficiency (Tan et al., 2014b). Previous study showed that exposure of chicken spleen macrophages to IBD virus induced the production of NO. Therefore, we presume that IBDV inoculated chickens may need more Arg than un-inoculated chickens to produce NO for immune defense.

Arg is an important immune-modulating nutrient in...
chickens, which has been reported to modulate circulating lymphocyte subpopulation and inflammatory cytokine expression in broiler chickens (Tan et al., 2014a, 2015). Deficiency of Arg suppressed the T-cell (Rodriguez et al., 2007), B-cell (De Jonge et al., 2002) and lymphoid organ (Kwak et al., 1999) development. Dietary Arg supplementation was reported to enhance the immune function of broilers (Tan et al., 2014b) and alleviate cyclophosphamide injection induced immunosuppression in weaned pigs (Han et al., 2009). Therefore, we hypothesize that dietary Arg supplementation may have a regulatory effect in attenuating IBD vaccine (IBDV) inoculation induced immunosuppression by enhancing immune functions in chickens. Based on the foregoing, the present study was conducted to evaluate the regulatory effects of dietary Arg supplementation on immune functions of immunosuppressive broiler chickens.

**Materials and Methods**

**Experimental Design**

The broiler management protocol for this study was approved by the China Agricultural University Animal Care and Use Committee. Five hundred, 1-day-old female broilers (Ross 308) were randomly assigned to 10 treatments, each treatment consisted of 5 replicates of 10 birds each. The experiment was designed as a 2 × 5 factorial arrangement with 2 challenge status factors (control: intramuscular inoculation of saline; IBDV: intramuscular inoculation of IBDV) and 5 dietary Arg concentrations (analyzed): 9.90, 13.9, 17.6, 21.3, and 25.3 g/kg.

**Chemical Analysis of Diet**

Before starting the experiment, all protein-containing dietary ingredients were analyzed for crude protein and amino acid concentration. Then, experimental diets were formulated using these analyzed values. Nitrogen was measured using a Leco model FP 2000 N combustion analyzer (Leco Corp., St. Joseph, MI) and data were expressed on a CP basis using 6.25 as a conversion factor. To analyze dietary amino acid composition, diet samples were hydrolyzed with 6 M HCl at 100°C for 24 h, and the amino acid concentrations in the hydrolysate were determined by HPLC (AOAC International, 2000; method 982.30 E (a, b, c)). Composition of the basal diets and nutrient concentrations for starter (d 1 to 21) are shown in Table 1.

### Table 1. Basal diet ingredients and nutrient composition (g/Kg dry diet)

| Ingredients (g/kg) | 9.90 g/kg | 13.9 g/kg | 17.6 g/kg | 21.3 g/kg | 25.3 g/kg |
|-------------------|-----------|-----------|-----------|-----------|-----------|
| Analyzed arginine concentration |           |           |           |           |           |
| Corn              | 580.8     | 580.8     | 580.8     | 580.8     | 580.8     |
| Soybean meal      | 110.0     | 110.0     | 110.0     | 110.0     | 110.0     |
| Canola meal       | 80.0      | 80.0      | 80.0      | 80.0      | 80.0      |
| Corn gluten meal  | 136.0     | 136.0     | 136.0     | 136.0     | 136.0     |
| Soybean oil       | 25.0      | 25.0      | 25.0      | 25.0      | 25.0      |
| Limestone         | 13.3      | 13.3      | 13.3      | 13.3      | 13.3      |
| Calcium hydrophosphate | 18.5     | 18.5      | 18.5      | 18.5      | 18.5      |
| L-Lysine-HCL      | 5.5       | 5.5       | 5.5       | 5.5       | 5.5       |
| DL-Methionine     | 0.9       | 0.9       | 0.9       | 0.9       | 0.9       |
| L-Threonine       | 0.1       | 0.1       | 0.1       | 0.1       | 0.1       |
| L-Tryptophan      | 0.5       | 0.5       | 0.5       | 0.5       | 0.5       |
| Choline chloride  | 2.5       | 2.5       | 2.5       | 2.5       | 2.5       |
| Sodium chloride   | 3.0       | 3.0       | 3.0       | 3.0       | 3.0       |
| L-Arginine        | 0.0       | 0.5       | 10.2      | 15.3      | 20.4      |
| L-Alanine         | 20.4      | 15.3      | 10.2      | 5.1       | 0.0       |
| Vitamin / trace mineral premix | 3.5   | 3.5       | 3.5       | 3.5       | 3.5       |
| Total             | 1000      | 1000      | 1000      | 1000      | 1000      |
| Nutritional value |           |           |           |           |           |
| ME (Mcal/kg)²     | 2.98      | 2.98      | 2.98      | 2.98      | 2.98      |
| Crude protein³    | 202.8     | 202.7     | 202.7     | 202.5     | 202.7     |
| Calcium²          | 10.0      | 10.0      | 10.0      | 10.0      | 10.0      |
| Non-phytate phosphorus² | 4.5     | 4.5       | 4.5       | 4.5       | 4.5       |
| Lysine³           | 11.0      | 11.0      | 10.9      | 10.9      | 11.0      |
| Methionine³       | 4.6       | 4.6       | 4.5       | 4.5       | 4.5       |
| Arginine³         | 9.90      | 13.9      | 17.6      | 21.3      | 25.3      |

¹ supplied the following (per kg complete diet): Cu, 8 mg; Zn, 75 mg; Fe, 80 mg; Mn, 100 mg; Se, 0.15 mg; I, 0.35 mg; vitamin A, 12500 IU; vitamin D₃, 2500 IU; vitamin E, 30 IU; vitamin K₃, 2.65 mg; thiamine, 2 mg; riboflavin, 6 mg; vitamin B₁₂, 0.025 mg; biotin, 0.0325 mg; folic acid, 1.25 mg; pantothenic acid, 12 mg; niacin, 50 mg.

² Calculated values.

³ Analyzed values.
IBDV Infection Model

The live intermediate IBDV (vaccine strain B87) was purchased from Harbin Veterinary Research Institute, Harbin, China. The vaccine virus was diluted using sterile saline solution (0.9%) with a titer of $5 \times 10^6$ egg infectious dose (EID) per ml. On d 14, the chickens in IBDV vaccinated group were intramuscularly inoculated with 200 μL of the intermediate strain of IBDV; chickens from the control group were sham-inoculated with an equal volume of sterile saline solution.

Measurements, Sampling, and Analyses

Growth Performance. Birds and feed remaining in each pen were weighed at 21 d of age to determine body weight (BW) gain, feed intake (FI) and mortality-corrected feed conversion ratio (FCR) during the overall feeding period.

Relative lymphoid organ weights. At 21 d of age, 1 bird from each replicate was killed by exsanguination while under deep anesthesia by an intraperitoneal injection of sodium pentobarbital (30 mg/kg of BW). Thymus, bursa of Fabricius, and spleen were then precisely removed and weighed. Relative lymphoid organ weights were calculated as lymphoid organ weight divided by BW.

Serum immunoglobulin A (IgA) and lysozyme concentration. At 21 d of age, 1 bird from each replicate was randomly selected to collect peripheral blood from the wing vein and the blood was centrifuged at 3500 $\times$ g for 10 min at 4°C. The serum was stored at $-20\degree$C until assay. Serum lysozyme activity was determined using a lysozyme kit (Jiancheng Bioengineering Institute, Nanjing, China) as previously described (Wu et al., 2007). Serum IgA concentration was determined using commercial IgA ELISA kit (Bethyl Laboratories, Montgomery, TX).

Isolation of peripheral blood mononuclear cells (PBMC). The separation of PBMC by Ficol density centrifugation was performed as described previously (Long et al., 2011). Briefly, the peripheral blood was carefully layered on top of the density-gradient centrifugation medium (density = 1.077; HaoYang Biological Manufacture Co. Ltd., Tianjin, China) in tubes (1:1), the tubes were centrifuged at 200 $\times$ g for 30 min. After centrifugation, the PBMC were collected at the plasma-ficoll interface and were washed 3 times in RPMI 1640 medium (include 5.0% inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES).

Hydrogen peroxide (H$_2$O$_2$) and NO production of PBMC. PBMC were resuspended in RPMI 1640 medium, $2 \times 10^6$ cells per well were seeded on 96 well-culture plates in the presence of concanavalin A (Con A, 5 μg/ml, Sigma, St. Louis, MO), incubated for 24 h at 37°C in a 5% CO$_2$ atmosphere. Culture supernatants were collected, the NO (nitrate + nitrite) and H$_2$O$_2$ concentration was determined by NO and H$_2$O$_2$ kits (Jiancheng Bioengineering Institute, Nanjing, China) as previously described (Lu et al., 2005).

Phagocytosis of neutral red by PBMC. At 21 d, the phagocytosis of neutral red by PBMC was measured according to a previous report (Zhang and Guo, 2008). A 200 μL aliquot of cell suspension ($2 \times 10^6$ cells per milliliter) was seeded on 96 well-plates. After incubation at 37°C in a 5% CO$_2$ atmosphere for 2 h, the monocytes were adhered on the surfaces of plate (Pertoft et al., 1980). Then the culture medium was discarded, 200 μL of 0.7% neutral red was added in the well and incubated for another 1 h. Cells were then washed with D-Hank’s medium for five times and incubated with cell lysis solution (1 mol/L acetic acid: ethanol=1:1) overnight at 4°C. The absorbance was recorded using an automated ELISA reader (model 550 Microplate Reader, Bio-Rad Pacific Ltd., Hong Kong, China) at wavelength of 570 nm and D-Hank’s served as blank, optical density (OD) value was recorded.

PBMC proliferation. At 21 d, a 190 μL aliquot of PBMC suspension ($2 \times 10^6$ cells per milliliter) were seeded on 96-well culture plates with either 10 μL of lymphocyte mitogen Concanavalin A (Con A) or Escherichia coli lipopolysaccharide (LPS, serotype 0111:B4, Sigma, St. Louis, MO) added to each well to provide a final concentration of 45 μg/mL Con A or 25 μg/LPS, the plates were incubated at 37°C in a 5% CO$_2$ atmosphere. After 72 h, 10 μL of cell counting kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD) reagent were added to each well and the plate was incubated for another 4 h. The optical density was then measured at a test wavelength of 450 nm and a reference 630 nm. Stimulation index (SI) was calculated as the OD value of stimulated culture divided by the OD value of non-stimulated cultures.

Serum IBDV and Newcastle disease virus (NDV) specific antibodies measurement. All chickens were vaccinated with NDV-IV strain vaccine (Harbin Veterinary Research Institute, Harbin, China) through intranasal and intraocular administration on d 9. Serum IBDV and NDV specific antibodies were analyzed using the IBDV Antibody Test Kit and NDV Antibody Test Kit (Idexx Laboratories, Westbrook, ME, USA) respectively.

Statistical Analyses

Data were subjected to ANOVA analysis using the GLM procedures of SAS 9.1 (SAS Institute Inc., Cary, NC) as 5 x 2 factorial arrangement of treatments that included Arg concentration and IBDV inoculation as the main effects and their respective interactions, $P \leq 0.05$ value was considered significant, and $0.05 < P \leq 0.10$ was considered a trend towards significance. The pen was used as the experimental unit. A polynomial contrasts were used to determine linear and quadratic responses of the main effect means to dietary Arg concentration. Additionally, the Arg requirement for optimal growth performance and immune functions of each block (5 Arg supplemental groups each block that were 9.90, 13.9, 17.6, 21.3, and 25.3 g/kg Arg supplementation; 10 blocks total) was estimated using quadratic regression model respectively within each replicate of dietary Arg concentrations. Subsequently, the 5 replicate requirement concentration from each of the control and challenged treatments were analyzed by ANOVA. The differences between group means were compared using Tukey’s multiple range tests, the results were expressed as mean±SE.
Results

Growth Performance and Lymphoid Organ Development

Growth performance and relative lymphoid organ weights data was shown in Table 2. The IBDV inoculation exerts no significant ($P>0.05$) effects on growth performance and relative lymphoid organ weights of chickens. Increasing Arg concentration linearly and quadratically increased ($P<0.05$) the BW gain, linearly and quadratically decreased ($P<0.05$) the FCR, and tended to decrease the FI linearly ($P=0.085$). No significant ($P>0.05$) differences was observed for relative weight of bursa of the fabricius, spleen and thymus in the present study.

Immune Response

Serum IgA and lysozyme concentration. As shown in Table 3, IBDV inoculation lowered serum lysozyme and IgA concentration ($P<0.05$). Increasing Arg concentrations linearly($P<0.05$) increased serum lysozyme concentration. Serum IgA concentration quadratically($P<0.05$) responded with increasing Arg concentration that increasing towards the middle level of Arg supplementation (17.6 g/kg) while decreasing towards the highest level of Arg (25.3 g/kg) supplementation. The interaction between IBDV inoculation and dietary Arg concentration tended to be significant ($P=0.062$) for serum IgA concentration, while no significant ($P>0.05$) interaction was observed for serum lysozyme concentration.

Proliferation and phagocytosis of PBMC. As shown in Table 3, IBDV inoculation significantly($P<0.05$) reduced the PBMC proliferation stimulated by ConA, and tended to increase ($P=0.06$) PBMC phagocytic activity. Increasing Arg concentration linearly and quadratically ($P<0.05$) increased the PBMC proliferation stimulated by LPS. No interaction exists between IBDV inoculation and dietary Arg concentration on PBMC proliferation and phagocytic activity ($P>0.05$).

NO and $H_2O_2$ production of PBMC. As shown in Table 3, IBDV inoculation significantly ($P<0.05$) suppressed PBMC in vitro NO and $H_2O_2$ production. Increasing Arg concentration quadratically increased ($P<0.05$) PBMC in vitro NO production, and tended to increase PBMC in vitro $H_2O_2$ production quadratically ($P=0.074$). No significant interaction ($P>0.05$) between IBDV inoculation and dietary Arg concentration was observed for NO and $H_2O_2$ production.

Antibody titers to NDV and IBDV. As shown in Table 3, IBDV inoculation significantly depressed ($P<0.05$) NDV antibody (NDV-Ab) titers, increasing Arg concentration significantly($P<0.05$) affected NDV-Ab titers that peaking towards the middle level of Arg (17.5 g/kg) supplementation and decreasing towards the highest level of Arg (25.3 g/kg) supplementation. No significant interaction ($P>0.05$) between IBDV inoculation and dietary Arg concentration was observed for NDV-Ab titers. As shown in Fig. 1, serum anti-IBDV antibody titer tended to be increased quadratically ($P=0.06$) with increasing Arg concentration. In the control groups, all the serum samples were negative for the development of anti-IBDV antibodies (data not shown).

Table 2. Influence of varying dietary arginine (Arg) concentrations on growth performance and relative lymphoid organ weights of broilers (% of live BW) with or without Infectious bursal disease vaccine (IBDV) challenge

| Main effect means | BW gain$^1$, (kg/bird) | Feed intake$^1$, (Kg/bird) | Feed conversion ratio$^1$, (kg /kg) | Bursa of Fabricius$^2$, % of BW | Spleen$^2$, % of BW | Thymus$^2$, % of BW |
|-------------------|------------------------|----------------------------|-------------------------------|-------------------------------|-----------------|-----------------|
| Control           | - 0.53                 | 0.91                      | 1.70                         | 0.174                        | 0.114           | 0.216           |
| IBDV inoculation  | + 0.54                 | 0.95                      | 1.72                         | 0.179                        | 0.107           | 0.228           |
| Arg level         |                         |                           |                               |                               |                 |                 |
| 9.9 g/kg          | 0.50$^a$               | 0.96                      | 1.94$^a$                     | 0.183                        | 0.118           | 0.219           |
| 13.9 g/kg         | 0.57$^a$               | 0.94                      | 1.64$^{bc}$                  | 0.181                        | 0.123           | 0.236           |
| 17.6 g/kg         | 0.55$^{ab}$            | 0.94                      | 1.71$^b$                     | 0.166                        | 0.109           | 0.230           |
| 21.3 g/kg         | 0.54$^{ab}$            | 0.85                      | 1.57$^{bc}$                  | 0.192                        | 0.095           | 0.206           |
| 25.3 g/kg         | 0.53$^b$               | 0.91                      | 1.72$^{bc}$                  | 0.158                        | 0.107           | 0.218           |
| SEM               | 0.01                   | 0.01                      | 0.04                         | 0.004                        | 0.003           | 0.004           |
| Source of variation$^3$ |                   |                           |                               |                               |                 |                 |
| IBDV              | 0.907                  | 0.486                     | 0.171                        | 0.622                        | 0.326           | 0.312           |
| Arg               | <0.001                 | 0.190                     | <0.001                       | 0.518                        | 0.187           | 0.530           |
| IBDV×Arg          | 0.277                  | 0.168                     | 0.580                        | 0.269                        | 0.841           | 0.692           |
| Contrast / IBDV$^4$ |                       |                           |                               |                               |                 |                 |
| Linear            | 0.032                  | 0.085                     | 0.001                        | 0.466                        | 0.051           | 0.437           |
| Quadratic         | 0.034                  | 0.626                     | 0.011                        | 0.871                        | 0.635           | 0.559           |

$^1$Means represent 5 cages per treatment, 10 birds per cage.

$^2$Means represent 5 cages per treatment, 1 bird per cage.

$^3$Factorial analysis based on analyzed Arg concentration.

$^4$Polynomial contrasts.
As shown in Table 4, IBDV inoculation increased \((P<0.05)\) the Arg requirement of chickens for the lowest FCR from 16.0±1.3 to 18.9±0.5 in quadratic model. No significant affect was observed on BWG \((P>0.05)\). The Arg requirement of chickens from IBDV inoculated groups \((17.5\pm0.6)\) for the highest serum IgA concentration tended to be higher \((P=0.07)\) than those in control groups \((16.1\pm0.6)\); the Arg requirement of chickens from IBDV inoculated groups \((19.8\pm2.1)\) for the highest PBMC proliferation (stimulated by LPS) tended to be higher \((P=0.08)\) than those in control groups \((16.3\pm0.8)\). No significant effect was observed for serum lysozyme concentration \((P>0.05)\).

**Discussion**

Since Arg is an important immune-modulating nutrient for broiler chickens \((Tan et al., 2014b, 2015)\), the object of this study was to test hypothesis that dietary Arg supplementation may regulate the immune function of IBDV inoculated chickens. IBD virus infection was reported to cause severe immunosuppression \((Saif, 1991)\), similarly, IBDV inoculation resulted in immunosuppression in the present study, which was illustrated by the decrease of serum lysozyme and IgA concentration, mitogen-stimulated PBMC proliferation \((LPS)\), PBMC in vitro NO and \(H_2O_2\) production, and serum NDV-Ab titers. Nutritional strategy, such as conjugated linoleic acid supplementation, has been used to alleviate IBDV-induced immunosuppression \((Long et al., 2011)\). Arg is an important immune-modulating nutrient, which has been reported to enhance the immune function of broilers \((Jahanian, 2009)\) that may potentially alleviate IBDV-inoculation induced immunosuppression.

Amino acid requirements of chickens undergoing subclinical microbial infection \((Star et al., 2012)\) and immunological stress \((Klasing and Barnes, 1988)\) are different from those in healthy status. Previous studies showed that additional amount of Arg would be needed for immunological and connective tissue challenges in broilers \((Corzo et al., 2003)\), and the Arg requirement for the highest immune functions.
(13.4 g/kg) is higher than those for maximum growth performance (12.6 g/kg) and FCR (12.9 g/kg) in broilers (Jahanian, 2009). As shown in the current study, the estimated Arg allowance of IBDV inoculated chickens for the lowest FCR was higher than those in control groups, and the estimated Arg allowance for the highest serum IgA concentration and PBMC proliferation (stimulated by LPS) of IBDV inoculated groups tended to be higher than those in control groups, suggesting that additional amount of Arg supplementation may be needed for IBDV inoculated broilers. While, the effect of dietary Arg supplementation on immune response of chickens is influenced by multiple factors, such as balance among basic amino acids and immune status of broilers, which may influence the Arg requirement of broilers. Therefore, it is difficult to establish an optimal dose of Arg supplementation in the present study, further studies are needed to evaluate the relative optimal Arg allowance of chickens in different nutrient and immune status.

Dietary Arg deficiency has been reported to reduce thymus, spleen, and bursa of Fabricius weight (Kwak et al., 1999), while no difference was observed in the present study. Lysozyme is well known for its bactericidal function and the activation of complement system and phagocytes. Increasing Arg concentration increased serum lysozyme activity of broilers at 21 d, which was similar with a previous study (Costas et al., 2011). The serum lysozyme activity is not static and can be influenced by physiological and environmental factors. In the present study, the lysozyme activity was lower in IBDV inoculated groups, indicating that IBDV inoculation resulted in an immunosuppression. Serum IgA plays an important role in defending the host against pathogens. Dietary Arg supplementation was reported to increase mucosal IgA concentration in broilers (Tan et al., 2014b), which is similar with the result in this study. The regulation of Arg on IgA production was considered to be mediated by the regulation of either IgA secretion and/or IgA transportation that was associated with the regulation of IgA secreting cells and plgR mRNA expression respectively (Tan et al., 2014b; Zhu et al., 2012).

The PBMC function of chickens in the present study was evaluated by PBMC in vitro NO and H2O2 production, and in vitro proliferation (stimulated by LPS and Con A), which can be used to evaluating the innate immunity of chickens. NO, metabolite of Arg, has been recognized as one of the most versatile compounds in the immune system (Bogdan, 2001), which is an important immune effector of macrophage. NO is involved in the control of infectious diseases, autoimmune processes and chronic degenerative diseases, as well as inter- and intracellular signaling capacity (Bogdan, 2001). Hydrogen peroxide (H2O2) is generated in macrophages by H2O and superoxide that plays an important role not only in pathogen killing but also in the activation of neighboring lymphocytes (Reth, 2002). In the present study, dietary Arg supplementation enhanced in vitro NO production of PBMC, which was similar with a previous study (Costas et al., 2011). Meanwhile, increasing Arg concentration tended to increase in vitro H2O2 production of PBMC quadratically, indicating that dietary Arg supplementation had a regulatory effect on PBMC function. In the current study, a significant reduction in PBMC proliferation (stimulated by Con A) was observed in IBDV vaccinated groups, which may be due to IBDV inoculation induced immunosuppression. Meanwhile, PBMC proliferation (stimulated by LPS) was up-regulated by increasing Arg supplementation which agrees with previous finding (Tayade et al., 2006). These results suggested that dietary Arg supplementation may have enhanced the PBMC function of broilers.

Adaptive immunity was evaluated by antibody response to IBDV and NDV. The level of anti-IBDV antibody can be used to determine the protective ability against IBDV infection in broilers. In the present study, dietary Arg supplementation (21.3 g/kg) increased serum anti-IBDV antibody titers, suggesting that supplementation with 21.3 g/kg Arg may enhance the defensive immunity of broilers against IBDV infection. Due to IBDV inoculation, serum NDV-Ab

---

**Table 4. Arginine (Arg) requirement of broilers from control and Infectious bursal disease vaccine (IBDV) inoculated groups based on growth performance and immune parameters (1 to 21 d, g/kg)**

| Item                           | Control group1,2 | IBDV inoculated group1,2 | P-value |
|--------------------------------|------------------|--------------------------|---------|
| Growth performance             |                  |                          |         |
| BW gain                        | 16.4±0.6         | 16.7±0.1                 | 0.387   |
| Feed conversion ratio          | 16.0±1.3         | 18.9±0.5                 | 0.034   |
| Immune function                |                  |                          |         |
| lysozyme                       | 17.9±1.6         | 19.5±1.0                 | 0.212   |
| IgA                            | 16.1±0.6         | 17.5±0.6                 | 0.073   |
| PBMC proliferation (LPS)       | 16.3±0.8         | 19.8±2.1                 | 0.082   |

1 Means represent 5 cages per dietary Arg concentration, 10 birds per cage, according to 9.9, 13.9, 17.6, 21.3, and 25.3 g/kg analysed Arg concentration (analyzed).
2 Data are presented as means±SE.
3 PBMC=Peripheral blood mononuclear cells.
titer of chickens from IBDV inoculated groups was lower than un-inoculated groups, indicating that IBDV induced immunosuppression caused a suboptimal response to vaccination. Intriguingly, supplementation with high concentrations of Arg (21.3 and 25.3 g/kg) reduced serum NDV-Ab titers. Our previous study showed that high level of Arg supplementation (higher than 17.6 g/kg) suppressed the percentages of circulating and splenic B cells (Tan et al., 2013). Therefore, the reduction of serum NDV-Ab titers caused by high concentrations of Arg supplementation may be mediated by the reduction of B cell percentage. It is well-known that immune-modulating nutrients can be helpful as well as harmful if not utilized in moderation (Klasing 2007; Tan et al., 2014a). Thus, further studies are needed to obtain a better understanding on the regulatory effects of Arg on humoral immune functions of chickens.

Immunosuppression can be caused by many factors including infections and immunologic stress, resulting in decreased growth performance, infection resistance and immune functions (Han et al., 2009). Previous studies showed that IBDV inoculation causes immunosuppression (Long et al., 2011; Tan et al., 2015). In the present study, the broilers inoculated with IBDV had lower concentrations of serum lysozyme and IgA, NDV-Ab titer, in vitro PBMC NO and H2O2 production, and PBMC proliferation (stimulated by ConA) compared to un-inoculated broilers, indicating that IBDV inoculation caused either humoral or cellular immunosuppression. In the present study, increasing Arg concentration enhanced the immune function of IBDV inoculated chickens, which was evidenced by increased serum lysozyme and IgA concentrations, PBMC proliferation (LPS), and in vitro PBMC NO and H2O2 yield. These results suggested that dietary Arg supplementation may have a potential regulatory effect in alleviating either humoral or cellular immunosuppression caused by IBDV inoculation.

In conclusion, the present study demonstrated that dietary Arg supplementation partially alleviated IBDV inoculation-induced immunosuppression, which was evidenced by enhanced serum lysozyme and IgA concentrations, and PBMC functions.

Acknowledgments

This study was supported by the Ear-marked Fund for China Agriculture Research System (CARS-42-G13). We also thank China Scholarship Council (CSC) for partial financial support of this research.

References

Bogdan C. Nitric oxide and the immune response. Nature Immunology, 2: 907–916. 2001.

Corzo A, Moran E and Hoehler D. Arginine need of heavy broiler males: applying the ideal protein concept. Poultry Science, 82: 402–407. 2003.

Costas B, Conceicao LEC, Dias J, Novoa B, Figueras A and Afonso A. Dietary arginine and repeated handling increase disease resistance and modulate innate immune mechanisms of Senegalese sole (Solea senegalensis Kaup, 1858). Fish & Shellfish Immunology, 31: 838–847. 2011.

De Jonge WJ, Kwikkers KL, Velde AA, Van Deventer SJH, Nolte MA, Mebius RE, Ruijter JM, Lamers MC and Lamers WH. Arginine deficiency affects early B cell maturation and lymphoid organ development in transgenic mice. Journal of Clinical Investigation, 110: 1539–1548. 2002.

Han J, Liu Y, Fan W, Chao J, Hou Y, Yin Y, Zhu H, Meng G and Che Z. Dietary L-arginine supplementation alleviates immunosuppression induced by cyclophosphamide in weaned pigs. Amino Acids, 37: 643–651. 2009.

International A. Official methods of analysis of AOAC International. 17th ed. AOAC Int., Gaithersburg, MD. 2000.

Jahanian R. Immunological responses as affected by dietary protein and arginine concentrations in starting broiler chicks. Poultry Science, 88: 1818–1824. 2009.

Khatri M and Sharma JM. Infectious bursal disease virus infection induces macrophage activation via p38 MAPK and NF-κB pathways. Virus Research, 118: 70–77. 2006.

Klasing KC. Nutrition and the immune system. British poultry science, 48: 525–537. 2007.

Klasing KC and Barnes DM. Decreased amino acid requirements of growing chicks due to immunologic stress. Journal of nutrition, 118: 1158–1164. 1988.

Kwak H, Austic R and Dietert R. Influence of dietary arginine concentration on lymphoid organ growth in chickens. Poultry Science, 78: 1536–1541. 1999.

Long FY, Guo YM, Wang Z, Liu D, Zhang BK and Yang X. Conjugated linoleic acids alleviate infectious bursal disease virus-induced immunosuppression in broiler chickens. Poultry Science, 90: 1926–1933. 2011.

Lu P, Liu F, Wang CY, Chen DD, Yao Z, Tian Y, Zhang JH and Wu YH. Gender differences in hepatic ischemic reperfusion injury in rats are associated with endothelial cell nitric oxide synthase-derived nitric oxide. World Journal of Gastroenterology, 11: 3441–3445. 2005.

Luiking YC, Poeze M, Ramsay G and Deutz NEP. The role of arginine in infection and sepsis. Journal of Parenteral and Enteral Nutrition, 29: S70–S74. 2005.

Pertot H, Johnsson A, Warmegard B and Seljelid R. Separation of human monocytes on density gradients of Percoll®. Journal of Immunological Methods, 33: 221–229. 1980.

Rajput ZI, Xiao CW, Hu SH, Habib M and Soomro NA. Enhancement of immune responses to infectious bursal disease vaccine by supplement of an extract made from Momordica cochinchinensis (Lour.) Spreng. seeds. Poultry Science, 89: 1129–1135. 2010.

Reth M. Hydrogen peroxide as second messenger in lymphocyte activation. Nature Immunology, 3: 1129–1134. 2002.

Rodriguez PC, Quiceno DG and Ochoa AC. L-arginine availability regulates T-lymphocyte cell-cycle progression. Blood, 109: 1568–1573. 2007.

Saif YM. Immunosuppression induced by infectious bursal disease virus. Veterinary Immunology and Immunopathology, 30: 45–50. 1991.

Star L, Rovers M, Corrent E and Van der Klis JD. Threonine requirement of broiler chickens during subclinical intestinal Clostridium infection. Poultry Science, 91: 643–652, 2012.

Stechmiller JK, Childress B and Cowan L. Arginine supplementation and wound healing. Nutrition in Clinical Practice, 20: 52–61. 2005.

Tan J, Liu S, Guo Y, Applegate TJ and Eicher SD. Dietary L-arginine supplementation attenuates lipopolysaccharide-
induced inflammatory response in broiler chickens. British Journal of Nutrition, 111: 1394–1404. 2014a.

Tan J, Applegate TJ, Liu S, Guo Y and Eicher SD. Supplemental dietary L-arginine attenuates intestinal mucosal disruption during coccidial vaccine challenge in broiler chickens. British Journal of Nutrition, 112: 1098–1109. 2014b.

Tan JZ, Guo YM, Applegate TJ, Du EC and Zhao X. Dietary L-arginine modulates immunosuppression in broilers inoculated with an intermediate strain of infectious bursa disease virus. Journal of the Science of Food and Agriculture, 95: 126–135. 2015.

Tayade C, Jaiswal T, Mishra S and Koti M. L-Arginine stimulates immune response in chickens immunized with intermediate plus strain of infectious bursal disease vaccine. Vaccine, 24: 552–560. 2006.

Van Den Berg TP. Acute infectious bursal disease in poultry: a review. Avian Pathology, 29: 175–194. 2000.

Wu G, Yuan C, Shen M, Tang J, Gong Y, Li D, Sun F, Huang C and Han X. Immunological and biochemical parameters in carp (Cyprinus carpio) after Qompsell feed ingredients for long-term administration. Aquaculture Research, 38: 246–255. 2007.

Zhang L and Guo Y. Effects of liquid DL-2-hydroxy-4-methylthio butanoic acid on growth performance and immune responses in broiler chickens. Poultry Science, 87: 1370–1376. 2008.

Zhu H, Liu Y, Xie X, Huang J and Hou Y. Effect of L-arginine on intestinal mucosal immune barrier function in weaned pigs after Escherichia coli LPS challenge. Innate Immunity, 17: 242–252. 2012.