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Immunostimulatory Effects of Arginine in Broiler Chickens Challenged with Vaccine Strain of Infectious Bursal Disease Virus

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Abstract: Infectious Bursal Disease (IBD) continues to pose potential threat to poultry industry all over the world. The disease can spell disaster not only through its infection but also by break of immunity in chickens vaccinated for other diseases. On the other hand, arginine (Arg), a ubiquitous, semi-essential amino acid has emerged as an imunostimulant from variety of human and animal studies. In the present study, we demonstrate the stimulatory effects of Arg on systemic immune response in chickens challenged by orally administration of intermediate plus strain of IBD virus at 28 days of age. A corn-soybean meal based diet containing different levels of Arg (0, 0.67, 1.37, 2.07 and 2.77) for the starter, (0, 0.53, 1.1, 1.68 and 2.25) for the grower and (0, 0.52, 1.04, 1.56 and 2.08) for the finisher was used. In a completely randomized design with five treatments of five replicates each and 10 chickens per replicate, 250 Cobb500 male broiler chickens from 0-49 days of age were used. To measure the innate, cellular and humoral immunity indicators (interferon-α, interferon-γ, immunoglobulin G) at 27, 35, 42 and 49 days of age, serum samples from each replicate of treatments were collected and subjected to ELISA. The result showed that Arg supplementation in the chickens basal diets significantly increased the serum levels of interferon-α, interferon-γ, immunoglobulin G at 35, 42 and 49 days of age ($p<0.05$). The different levels of Arg at 27 days of age did not significantly affect interferon-α, interferon-γ, whereas Arg at 27 days of age significantly increased immunoglobulin G ($p<0.05$). These results revealed that arginine stimulates systematic immune response against intermediate plus strain of IBDV.

Key words: Arginine, immune response, immunity indicators, infectious bursal disease virus, broiler chicken

INTRODUCTION

Infectious Bursal Disease (IBD) or Gumboro disease caused by IBD virus is one of the most important immunosuppressive diseases affecting mainly young chickens and considered as a threat to commercial poultry (Lukert and Saif, 1997). Eradication of IBD virus is difficult because of its high stability in the environment. Therefore, the principal approach for prevention is effective vaccination against this disease. The current vaccines are killed or cell culture adapted live, attenuated virus. Depending on the level of attenuation and virulence, these live vaccines are further categorized as hot or intermediate plus, intermediate and mild. The most effective strategies to control IBD include vaccination of layer birds with inactivated oil-emulsified vaccines to provide maternal antibodies in chickens or immunization of young chickens with live attenuated vaccines followed with booster immunizations. After the emergence of very virulent IBDV, mild vaccines are generally ineffective and presence of maternal antibodies interferes with the efficacy of live vaccines. Another most important issue associated with live vaccines is immunosuppression, which may cause lesions similar to natural infection in vaccinated birds (Muller *et al*., 2003). Thus, there is a definite requirement for a suitable immunomodulator that can minimize the suppressive effects of live vaccines and amplify specific protective response. Arginine, a semi-essential dibasic amino acid has emerged as a regulator of many immunological and physiological processes. Arginine attracted initial experimental attention in various animal tumor models (Barbul *et al*., 1980) as a dietary supplement. The most promising immunostimulatory effects were observed in immunocompromised hosts after trauma, surgical stress or immunosuppression with HIV virus. Supplemental Arg

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stimulates the functional activities of different cell types including Natural Killer (NK) cells, macrophages, lymphokine activated killer cells, T and B cells (De Jonge *et al*., 2002). Two pathways of arginine metabolism have been identified as being critical to the immunomodulatory actions of arginine *in vivo*. First, the arginase pathway, in which Arg is converted to urea and ornithine, generating polyamines by the action of ornithine decarboxylase. This route of polyamine synthesis may be the mechanism whereby Arg augments lymphocyte mitogenesis. Induction of arginase has also been proposed as the effector pathway in arginine-dependent macrophage-mediated tumor cell cytotoxicity (Currie, 1978). Second, arginine is the sole substrate for nitric oxide synthesis in biological systems. Nitric oxide is synthesized from arginine by nitric oxide synthase resulting in the formation of nitric oxide and citrulline. Nitric oxide is a ubiquitous molecule with important roles in the maintenance of vascular tone, coagulation, the immune system and the gastrointestinal tract and has been implicated as a factor in disease states as diverse as sepsis, hypertension and cirrhosis (Billiar, 1995). Although, IBDV primarily impairs humoral immune response, sever immunosupression is the result of downregulation of T cells and macrophages. These two cells are the main producers of cytokines involved in innate and cell mediated immunities. Cytokines are low-molecular-weight proteins or glycoproteins that are primarily produced by immune cells and are able to direct and regulate immune responses. Similar to mammalian Interferons (IFN), there are two types of chicken IFNs; types I and II. Type I IFN with antiviral activity, includes IFNα produced by monocytes and macrophages and IFNβ produced by fibroblast and epithelial cells. IFNγ, a type II IFN is produced by activated Natural Kill cells (NK) as well as T cells and considered to be a TH1-biased cytokine. Chicken IFNγ shares many biological properties with mammalian IFN-γ (Lowenthal *et al*., 2001). It activates macrophages to enhance their antimicrobicidal activities, up-regulates the expression of Major Histocompatibility Complex (MHC) I molecules to increase antigen presentation and induces secretion of other cytokines such as interleukin-2 (IL-2) and IL-12 to develop Cell-Mediated Immunity (CMI). IFNγ secreted by TH1 cells can also switch the antibody classes to support phagocytosis and fixation of complement (such as IgG2a in mouse) (Tuting *et al*., 1999). The sever immunosupression and pathological symptoms caused by IBDV infection, could be reduced by immunostimulator nutrients such as Arg in the chicken's basal diet. Therefore, the present study was aimed to investigate the effects of Arg on the overall immune response. The serum levels of IFNα, IFNγ and IgG were measured as the indicators of innate, Cell mediate and humoral immune responses, respectively.

MATERIALS AND METHODS

Five different dietary levels (A-E) of Arg for three age group periods (starter, grower and finisher) of broiler chickens were used. There were (Table 1) five levels for the starter (S) 0-21 days of age (S-A, 0-control; S-B, 0.67;

Table 1: Composition of experimental diets of broiler chickens during 0-49 days of age

	Starter (S)					Grower (G)					Finisher (F)				
Ingredients	S-A	S-B	S-C	S-D	S-E	G-A	G-B	G-C	G-D	G-E	F-A	F-B	F-C	F-D	F-E
Corn grain	54.4	54.4	54.4	54.4	54.4	67.9	67.9	67.9	67.9	67.9	71.1	71.1	71.1	71.1	71.1
Soybean meal	35.3	35.3	35.3	35.3	35.3	22.6	22.6	22.6	22.6	22.6	20.03	20.03	20.03	20.03	20.03
Fish meal	1.09	1.09	1.09	1.09	1.09	4.3	4.3	4.3	4.3	4.3	2.5	2.5	2.5	2.5	2.5
Dicalcium phosphate	1.35	1.35	1.35	1.35	1.35	0.54	0.54	0.54	0.54	0.54	0.55	0.55	0.55	0.55	0.55
Limestone	1.17	1.17	1.17	1.17	1.17	1.19	1.19	1.19	1.19	1.19	1.12	1.12	1.12	1.12	1.12
Vitamin-mineral mix[1]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Vegetable oil	2.8	2.8	2.8	2.8	2.8	-	-	-	-	-	-	-	-	-	-
Salt	0.4	0.4	0.4	0.4	0.4	0.24	0.24	0.24	0.24	0.24	0.2	0.2	0.2	0.2	0.2
DL-Methionine	0.14	0.14	0.14	0.14	0.14	-	-	-	-	-	-	-	-	-	-
Arginine	0	0.67	1.37	2.07	2.77	0	0.53	1.1	1.68	2.25	0	0.52	1.04	1.56	2.08
Wheat bran	2.8	2.13	1.43	0.73	0.003	2.6	2.07	1.5	0.92	0.35	3.99	3.47	2.95	2.43	1.91
Calculated analysis	2900	2900	2900	2900	2900	2900	2900	2900	2900	2900	2900	2900	2900	2900	2900
ME[2] (kcal kg^{-1})															
Crude protein (%)	20.8	20.8	20.8	20.8	20.8	18.2	18.2	18.2	18.2	18.2	16.3	16.3	16.3	16.3	16.3
Crude fiber (%)	3.70	3.70	3.70	3.70	3.70	3.20	3.20	3.20	3.20	3.20	3.05	3.05	3.05	3.05	3.05
Linoleic (%)	2.2	2.2	2.2	2.2	2.2	1.6	1.6	1.6	1.6	1.6	1.7	1.7	1.7	1.7	1.7
Ca (%)	0.91	0.91	0.91	0.91	0.91	0.82	0.82	0.82	0.82	0.82	0.72	0.72	0.72	0.72	0.72
Available. P (%)	0.41	0.41	0.41	0.41	0.41	0.32	0.32	0.32	0.32	0.32	0.27	0.27	0.27	0.27	0.27
Na (%)	0.18	0.18	0.18	0.18	0.18	0.14	0.14	0.14	0.14	0.14	0.11	0.11	0.11	0.11	0.11
Arginin (%)	1.34	2.01	2.68	3.35	4.02	1.10	1.65	2.20	2.75	3.30	1.00	1.50	2.00	2.50	3.00
Lysine (%)	1.15	1.15	1.15	1.15	1.15	1.00	1.00	1.00	1.00	1.00	0.85	0.85	0.85	0.85	0.85
Methionine+cystine (%)	0.82	0.82	0.82	0.82	0.82	0.65	0.65	0.65	0.65	0.65	0.58	0.58	0.58	0.58	0.58
Tryptophan (%)	0.30	0.30	0.30	0.30	0.30	0.24	0.24	0.24	0.24	0.24	0.21	0.21	0.21	0.21	0.21

[1]Supplied per kilogram of diet: vitamin A, 10,000 IU; vitamin D_3, 9790 IU; vitamin E, 121 IU; B_{12}, 20 μg; riboflavin, 4.4 mg; calcium pantothenate, 40 mg; niacin, 22 mg; choline, 840 mg; biotin, 30 μg; thiamine, 4 mg; zinc sulphate, 60 mg; manganese oxide, 60 mg; [2]Metabolizable energy

S-C, 1.37; S-D, 2.07 and S-E 2.77), five levels for the grower (G) 21-42 days of age (G-A, 0-control; G-B, 0.53; G-C, 1.1; G-D, 1.68 and G-E, 2.25) and five for the Finisher (F) 42-49 days of age (F-A, 0-control; F-B, 0.52; F-C, 1.04; F-D, 1.56 and F-E, 2.08). All diets met the National Research Council (1994) recommendations for broilers. One-day-old Cobb 500 male broiler chickens (250) were utilized in the experiment consisting of 5 treatments with 5 replicates and 10 chickens per replicate each. Birds were housed randomly in pen, so that initially each bird occupied approximately 0.11 m^2 of floor space. The pens were floor pens with wood litter. Birds were maintained under continuous light and the environmental temperature in the barn that was initially established on 31°C and was gradually reduced to 20°C by week 7. Feed and water were provided *ad libitum* throughout the experiment. Chickens were challenged by orally administration of intermediate plus strain of IBD virus (10-fold greater than normal vaccination doses) at 28 days of age. This strain was obtained from the poultry vaccine testing laboratory, Malaysian Vaccines and Pharmaceuticals. At 27 (Day Before Challenge: DBC), 35, 42 and 49 (Days After Challenge: DAC) days of age, two chicken was randomly selected from each replicate in each treatment and blood samples were collected from wing vein by Terumo Syringe with needle (0.7×32 mm). Blood samples (ten samples for each treatment) were centrifuged, serum was separated and stored at -20°C until use and analyzed in triplicate to assure that calculated and analyzed values were in agreement. Analysis of the immune response indicators including interferon α (INF α), interferon γ (INF γ), Immunoglobulin G (IgG) were determined using appropriate Enzyme Linked Immunosorbent Assay (ELIZA) (USCN, USA). INF α, INF γ and IgG were evaluated as indicators of the effects of orally administered intermediate plus strain of IBDV with or without Arg on the systemic innate, cell-mediated and humoral immune responses, respectively. ELISA was performed according to the manufacturer's instructions. Briefly, equal amounts (100 mL) of positive, negative controls and serum samples were added to the previously marked wells and the plates were incubated for 30 min at room temperature. After 3 washings, 100 mL of conjugate provided with the kit was added to each well and incubated for 30 min at room temperature. The plates were again washed and 100 mL of substrate solution was added and incubated for 15 min. Later, stop solution was added and plates were read at ELISA plate reader set at 450 nm. At 49 days of age bursas and spleens were removed, sections of bursas and spleens tissues from the inoculated and control chickens were fixed in 10% formalin solution and stained with hematoxylin and eosin (H and E). Sections of bursas and spleens were examined microscopically and photographed by digital camera equipment (LEICA DMIRB, Leica Microsystems Wetzlar, Germany).

A completely randomized experimental design was used. All data were statistically analyzed using the General Linear Models (GLM) procedure of SAS software (SAS Institute, 1996) was employed for the analysis of variance. Duncan (1955)`s multiple range test was used to determine differences among treatment means. Means were considered different at $p<0.05$.

RESULTS AND DISCUSSION

Effects of different levels of Arg on the immune system indicators in broiler chickens are shown in Table 2-4. Arginine supplementation into the basal diets significantly increased interferon-α, interferon-γ, immunoglobulin G at 35, 42 and 49 DAC days of age of

Table 2: Effects of arginine on the serum level of interferon-α in broiler chickens (pg mL^{-1})

Arg groups	27 days	35 days	42 days	49 days
A	1131.1	870.7^{c}	713.8^{a}	844.3^{a}
B	1132.0	984.1^{b}	945.0^{ab}	972.7^{a}
C	1153.7	1302.9^{ab}	972.0^{ab}	1067.5^{ab}
D	1156.8	1515.6^{a}	1123.7^{b}	1110.3^{ab}
E	1156.8	1379.8^{a}	1110.7^{b}	1071.0^{b}
SE	42.510	113.24	72.630	79.190
p-value	0.9800	0.0050	0.0100	0.0160
R^2	0.0036	0.2800	0.2200	0.0700

$^{a-c}$Means in each column with different superscripts are significantly different ($p<0.05$)

Table 3: Effects of arginine on the serum level of interferon-γ in broiler chickens (pg mL^{-1})

Arg groups	27 days	35 days	42 days	49 days
A	1205.5	731.3^{a}	1112.4^{a}	1159.1^{a}
B	1273.4	1585.8^{b}	2180.4^{b}	2359.9^{b}
C	1398.7	1700.4^{b}	2236.7^{b}	2409.9^{b}
D	1520.0	2078.7^{b}	2275.0^{b}	2485.3^{b}
E	1477.4	1863.3^{b}	2241.1^{b}	2482.6^{b}
SE	158.59	273.35	62.300	146.50
p-value	0.2100	0.0080	0.0001	0.0001
R^2	0.0900	0.2400	0.8600	0.8800

^{a,b}Means in each column with different superscripts are significantly different ($p<0.05$)

Table 4: Effects of arginine on the serum level of immunoglobulin G in broiler chickens (ug mL^{-1})

Arg groups	27 days	35 days	42 days	49 days
A	26.0^{a}	25.90^{c}	39.70^{c}	39.80^{a}
B	35.5^{b}	34.30^{a}	54.00^{c}	43.40^{a}
C	36.3^{b}	37.90^{ab}	75.30^{b}	63.90^{ab}
D	38.8^{b}	55.90^{ab}	97.70^{ab}	78.10^{b}
E	37.2^{b}	48.90^{b}	90.10^{a}	75.40^{b}
SE	2.41	8.200	13.00	13.26
p-value	0.01	0.016	0.020	0.017
R^2	0.21	0.200	0.160	0.190

$^{a-c}$Means in each column with different superscripts are significantly different ($p<0.05$)

chickens ($p<0.05$). Interestingly, the different levels of Arg at 27 DBC day of age did not significantly affect interferon-α, interferon-γ, whereas Arg at 27 DBC day of age significantly increased immunoglobulin G ($p<0.05$).

All the unimmunized control birds succumbed to infection by intermediate plus challenge virus. These chickens showed typical signs of IBD, as such some of them had gross bursal lesions characterized by pale color edema, point bleeding and significantly lower bursa to body weight ratio ($p<0.05$) compared to other chickens. Spleen enlargement was also significantly higher in control group than the other groups. The bursal lesion scores are shown in Table 5 and 6 and histopathological lesion scores are shown in Fig. 1. The spleen lesion scores are shown in Table 7 and 8 and histological lesion scores are shown in Fig. 2. As these data confirmed sever lesions were observed in group A whose diet was not supplemented with Arg and with the increase of Arg the lesions severity decreased (Fig. 1 and 2).

The results revealed that with an increase in Arg level, INF α, INF γ and IgG were enhanced linearly. This linear enhancement was continuous up to group D but in group E, a negative deflection was observed. As a whole, the highest level of all immunity indicators, were induced on DAC and DBC of age that is fitted in group D. Although, no statically responses were observed for INF α, INF γ at 27 DBC, trends of their improvement were observed as Arg level was increased in diet.

Mass cull of chickens caused by Immunosuppressive effects of IBDV threatens the poultry industry worldwide. The main objective of the study was to evaluate high levels of Arg as an immunomodulator of systemic immunity. It is believed that Arg can reduce the immunosuppressive effects of live hot vaccine and amplify specific protective immune response against IBD (Mo *et al*., 2001). The result showed that dietary Arg increased the serum levels of INF α, INF γ and IgG. Two percentage of Arg supplementation in feed was safe and free from any side effects (Tayada *et al*., 2006). The enhancement of immune response was further confirmed by histopathological examination of bursa and spleen.

Innate immunity is anatomically positioned to serve as the first line of defense against variety of pathogens such as viral infections. One of the elements of innate immunity is IFN-α, which is quickly upregulated after infection with viruses (Kawai and Akira, 2006). Mo *et al*. (2001) showed that recombinant IFN-α suppressed IBDV and Newcastle disease infection in chickens. This finding is in line with our result in which the increase of IFNα in intermediate plus + Arg group reduced the clinical signs of the infected chickens. In chickens, cellular innate immunity constitutes NK and macrophages (Gobel *et al*., 2001). Arginine supplementation has been shown to enhance NK (Brittenden *et al*., 1994). Moreover, Reynolds *et al*. (1990) observed interferon and macrophage cytotoxicity enhancement with Arg enriched diet.

IFN-γ is associated with adaptive immune responses and as such would be expected to be upregulated later in the immune response (Takaoka and Kani, 2006). IFN-γ is a pleiotropic cytokine secreted by Natural Killer (NK) cells and many T cells. This cytokine has the ability to direct the balance of TH cells to TH1 and induce cellular immune responses which leads to the lysis of virus-infected or tumor cells (Urban *et al*., 1996).

Table 5: Effects of arginine on the bursa lesion scores in broiler chickens

Arg groups	49 days
A	5
B	3
C	2
D	1
E	1

Table 6: Lesion scoring for Bursa of fabricus

Score	Grade	Lesions
0	Normal	Normal or non pathognomic lesions
1	Mild	Slightly degenerative changes at the lining epithelium and the medullary region of lymphoid follicles
2	Mild to moderate	Mild to moderate degenerative and or necrotic events of lymphoid cells in the medullas of some lymphoid follicles-infiltration of some acute-sub acute inflammatory in the oedematous connective tissue
3	Moderate	Moderate necrosis of lymphoid follicles involving both medullas (mostly) and cortex (occasionally). Pyknosis as early nuclear change was observed and the interstitial connective tissue is invade by hetrophils, macrophages and plasma cell together with few escaped erythrocytes
4	Moderate to severe	Karryorrhexis and karryolysis as advance nuclear changes is seen in some necrotic cells within lymphoid follicles together with moderate depletion of lymphoid cells in these follicles. Bursa with infectious bursists presenting cycts and lymphoid follicle hypotrophy. The interstitial connective tissue spaces were infiltrated with sub acute-chronic inflammatory cells (lymphocytes, plasma cells, macrophages, some few fibroblasta). The intra and extra follicular area might be hyperaemic and/or hemorrhagic. The epithelial lining, were thickened and some times appear corrugated together with increased number of goblet cells
5	Severe	Frank sever necrosis involving both cortex and medulla. Fibrinous exudates and necrotic cheesy depress were frequently observed. Oedematous connective tissue was obvious that infiltrated by sub acute-chronic inflammatory cells especially macrophages, lymphocytes, plasma cells and fibroblasts. The epithelial lining of the burses was thickend and vaculat of due to the prominence of goblet cells

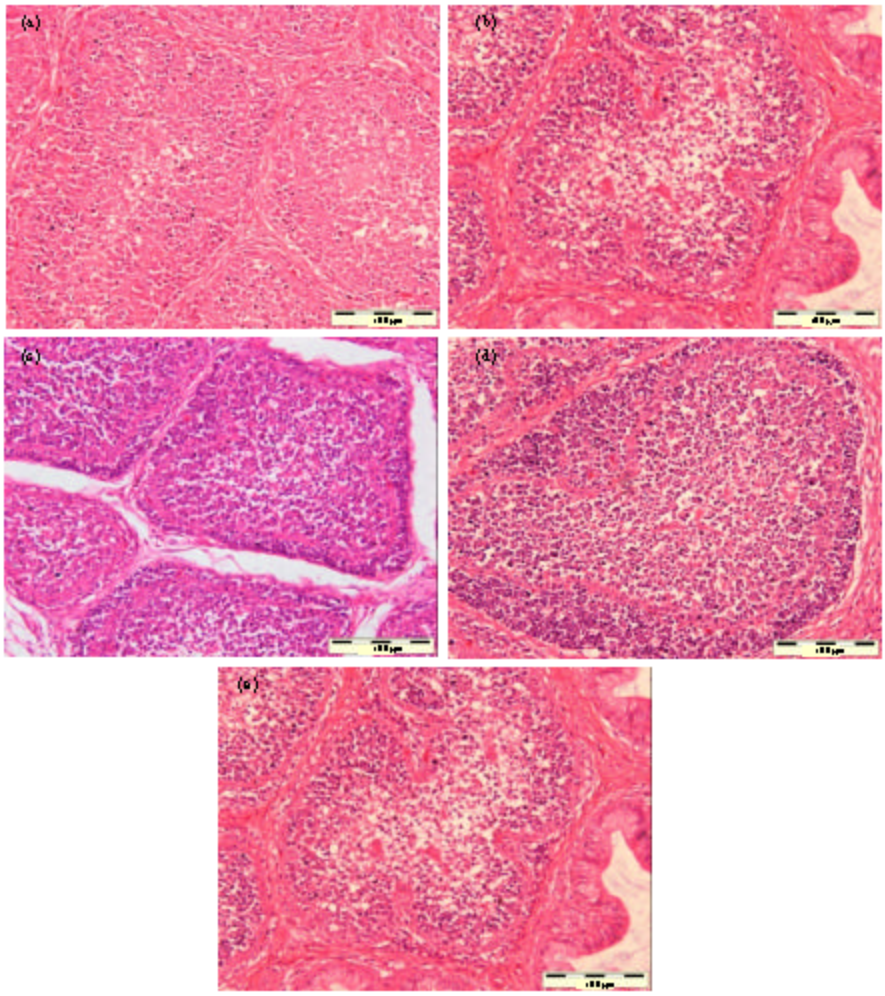

Fig. 1: Histopathology slides of Bursa in different groups

It has been reported that chicken IFN-γ level is increased before the appearance of the first clinical signs. During the acute phase of IBD infection an increase in the levels of circulating cytokines like chicken IFN-γ and TNF-α occur as demonstrated by the capture ELISA and cytotoxic bioassay, respectively. The increased levels of IFN-γ and TNF-α correlated with the progression of the disease and were highest in the animals that died of co infection (Saif, 1984). Co-administration of either IFN-γ protein or plasmid DNA encoding IFN-γ with DNA vaccine showed that the immune response was enhanced in felines (Flynn *et al*., 2000), ducks (Long *et al*., 2005) and chickens (Min *et al*., 2001) against challenge to feline immunodeficiency virus, hepatitis B virus and *Eimeria acervulina*, respectively. Consistent with these findings, the results showed that Arg supplementation into the basal diets significantly increased interferon-γ, at 35, 42 and 49 DAC days of age of chickens ($p<0.05$). Interestingly, the different levels of Arg at 27 DBC day of age did not significantly affect interferon-γ.

Table 7: Effects of arginine on the spleen lesion scores in broiler chickens

Arg groups	49 days
A	4
B	3
C	2
D	1
E	1

The levels of neutralizing antibodies determine the protection against IBDV in chickens (Van den Berg, 2000). Binding of these antibodies with virus forms immune complexes, which are removed by complement-mediated lysis (Rautenschlein *et al*., 2002). Similar to other findings (Tayada *et al*., 2006), we observed a strong antibody response in all groups; however antibody titers were significantly higher in challenged groups which

Table 8: Lesion scoring for Spleen

Score	Grade	Lesions
0	Normal	Normal histologic architecture without pathognomic lesion
1	Mild	Few splenic corpuscles revealed scattered lymphoid degeneration and necrosis in the white pulp, the germinal center and peripheral zones were indistinct with slight congestion of parenchymatous blood vessels
2	Moderate	Moderate lymphoid depletion due to (necrosis and apoptosis) in some splenic corpuscles and lymphatic cords of red pulp with moderate congestion and few petechial hemorrhagic area in the intstitial connective tissue together slight hamosiderosis
3	Moderate to severe	Moderate to sever lymphoid depletion in most splenic corpuscles (atrophy) and some lymphatic cords of red pulp with frank petechial hemorrhages and hemosiderin deposition
4	Severe	Frank loss or atrophy of splenic corpuscles with deposition of necrotic depress nests and petechial hemorrhage with infiltration of macrophages plasma cells and ectopic lymphocytes

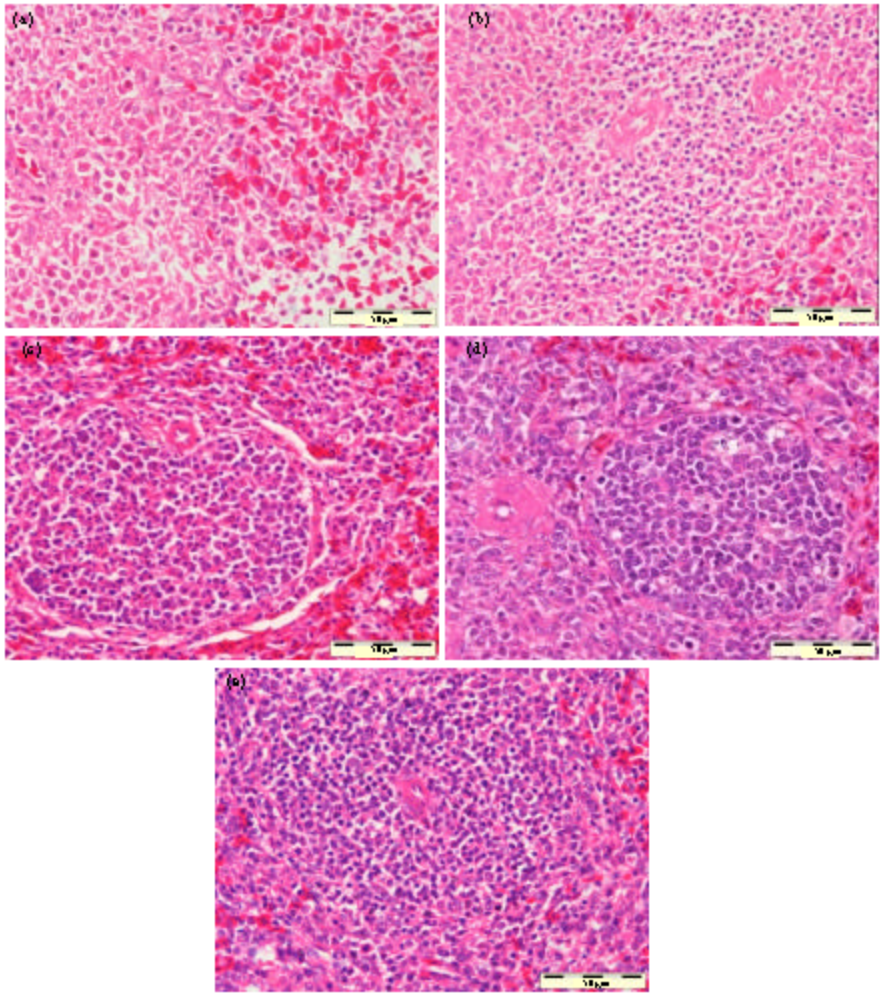

Fig. 2: Histopathology slides of the spleen in different groups

received Arg. The elevated antibody response and protection are attributed to the various immunoregulatory functions of Arg on the immune system. It is believed that Arg is required for the differentiation of pro B to pre B cells as well as being involved in the release of these cells from bone marrow (De Jonge *et al*., 2002).

Abdukalykova and Ruiz-Feria (2006) proved that high levels of Arg (2.2%) and higher-than-industry levels supplementation of vitamin E (80 IU kg^{-1}) had an important immunomodulation effect on the cell-and humoral-mediated immune responses of IBDV vaccinated broiler chickens by increasing the amount of T cells and B cells and the CD4+ and CD8+ lymphocyte subpopulations.

Regardless of vitamin E, the results obtained from this study not only are complementary to their findings but also reveals that innate immunity is significantly enhanced by high level of Arg.

CONCLUSION

To the knowledge, this is the first comprehensive study of the effects of high levels of Arg on innate, humoral and cellular immune responses on chickens challenged with intermediate plus strain of IBDV. As the results revealed 2.5 times of NRC level of Arg supplementation in feed was safe and free from any side effects as well as having the optimum effects on all mentioned parameters which led to the protection of chickens against the intermediate plus strain of IBDV. Therefore, Arg may be able to enhance the resistance of broilers to infectious diseases and may be useful in minimizing the stress associated with vaccination.

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