Effect of *In ovo* Injection of Critical Amino Acids on Pre- and Post-hatch Growth, Immunocompetence and Development of Digestive Organs in Broiler Chickens

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ABSTRACT : Two experiments were conducted to standardize *in ovo* injection of amino acids (AA) and to evaluate the effect of *in ovo* injection of limiting AA(s) on pre and post hatch growth performance, immune response and development of digestive organs. Combinations of essential and non-essential amino acids (Lys+Arg, Lys+Met+Cys, Thr+Gly+Ser, Ile+Leu+Val and Gly+Pro) were injected into 50 eggs in each treatment group at 14 d of embryonic age. Standardization of injection site, needle length and embryonic age revealed that when AA were injected in to the broad end of the egg with a 11 mm needle and at the narrow end with a 24 mm needle both at the 7th and 14th d of incubation there was poor hatchability. However, better hatchability was recorded when the AA were injected in the narrow end of the egg with a 11 mm needle and in the broad end with a 24 mm needle on the 14th d of incubation. The chick to egg weight ratio was higher ($p<0.018$) when AA were injected on the 14th d of incubation. When a combination of amino acids were injected a 63.6 or 63.2 g difference in body weight of bird at 21 d was recorded between uninjected control and Ile+Leu+Val or Gly+Pro group, respectively. Higher feed intake ($p<0.047$) was recorded in the AA injected groups and feed conversion ratio (FCR) was numerically better in Gly+Pro, Lys+Met+Cys AA injected groups than in the uninjected control. Significantly higher immune response to cell mediated ($p<0.033$) and humoral ($p<0.002$) immunity was observed in *in ovo* amino acid injected birds, especially in Lys+Met+Cys, Thr+Gly+Ser or Ile+leu+Val groups. The digestive organ weights at 21 d did not differ between specific AA injected groups and the uninjected control. *In ovo* injected amino acids may act as immunomodulators and their role in gastrointestinal development needs further research. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 4 : 524-531)***

Key Words : *In ovo* Injection, Amino Acids, Growth, Gastrointestinal Tract, Immune Response

INTRODUCTION

Albumen and yolk contain substantial amounts of amino acids for the developing embryo. A large fraction of egg protein consists of antibodies, that the hen produces during the immune response experienced at the time of egg laying (Losch et al., 1986). In normal circumstances these specific proteins are used for passive immunity until the neonate can mount an effective immune response (Brierley and Hemmings, 1956). Thus, early provision of nutrients not only affects immediate embryo survival and disease resistance, but also the ultimate attainment of genetic potential. *In ovo* injection of amino acids may also spare those antibodies from utilization as protein source during embryonic and neonate stage. During the early period of embryonic growth there is higher utilization of Gly, Pro, Lys and Arg. The proportion of Gly and Pro in the embryo increases with incubation time. Pro is synthesized from several AA, where as precursors of Gly are limited to only Thr and Ser (Ohta et al., 1999).

Injection site has a good relationship with the hatchability of AA injected into eggs (Ohta and Kidd, 2001, Ohta et al., 2002). AA injected on the 7th d of incubation increases the chick weight by 3.6% (Ohta et al., 1999,2001). Al-Murrani (1982) also reported a 11% higher chick weight in a AA injected group and such differences existed through out the growth period. By 56 d, the difference increased to 12.7%. Thr is closely associated with the digestive enzymes and mucous in the digestive tract of chicken (Yang et al., 1989). In pigs approximately 62% of dietary Thr was not recovered in the portal system, and 90% of the total Thr retained by the intestine and either used for mucin formation or was catabolized by enterocytes (Stoll et al., 1998). The Thr requirement for amylase synthesis is particularly high at approximately 11% of protein (Block et al., 1966).

The present investigation was conducted to study methods of injecting AA and the effect of *in ovo* injection of some critical amino acids on embryonic and post–hatch growth performance, immune response and development of digestive organ in chicks hatched from broiler breeder eggs.

MATERIALS AND METHODS

Experiment 1

Standardization of in ovo injection : A preliminary study was conducted to standardize the site of *in ovo* injection (broad and narrow end of egg), needle length (11 and 24 mm), embryonic age (7th and 14th d) and their affect on the

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Table 1. Concentration (mg/0.5 ml of sterile water) of critical AA used in *in ovo* injection

Control	T-1	T-2	T-3	T-4	T-5
No injection	Concentration of amino acids injected into egg (mg)				
	Lys-5.84	Lys-5.84	Thr-3.91	Ile-4.19	Gly-2.74
	Arg-5.01	Met-2.95	Gly-2.74	Leu-7.00	Pro-3.03
		Cys-1.70	Ser-5.97	Val-5.16	

hatchability of the egg. For this purpose, fertile eggs (n=340) were fumigated, weighed, distributed into 17 groups having 20 eggs each and placed group wise in a forced draft incubator. On 7th and 14th d of embryonic age, 4 treatments each were injected with AA solution (1-broad end and 24 mm, 2-narrow end and 24 mm, 3-broad end and 11 mm, and 4-narrow end and 11 mm needle length). The injection was carried out under laminar flow chamber, where the temperature of the chamber was maintained at 35°C. The *in ovo* injection of each treatment was completed within 15 minutes of taking out from the incubator and the eggs were returned back to the incubator to assess the effect on hatchability of fertile eggs and chick weight at hatch. One control group, which did not receive any injection, was also maintained in the incubator and on 7th d this group was also kept in laminar flow chamber for 15 minutes to equate the injectable environment. The AA injections were done through a pinhole made at the respective end of the egg under laminar flow. Immediately after the injection, the site was sealed with sterile paraffin and eggs were returned to the incubator. On 7th and 14th d of embryonic age, the other 8 groups were injected with 0.5 ml solution of Indian ink, through a pinhole into the developing embryo to assess the site of deposition of AA. Immediately after the injection, the shell of the respective ends of the egg was cut with a dental drill, and the eggshell and shell membranes of eggs were removed with forceps. The injection site was visually observed. The Indian ink deposition site was classified in to five groups: air cell, amniotic cavity, extra embryonic cavity, yolk sac and albumen. Deposition of AA solution, hatchability of fertile eggs, chick weight at hatch and chick weight to egg weight ratio were the assessing criteria.

On the 19th d the eggs were shifted to the hatcher and kept in the respective pedigree hatching boxes. On the 22nd d the chicks were weighed, wing banded and transferred to the battery brooders for a performance study.

Preparation of amino acid solution : The AA composition of an egg (Ohta et al., 2001) was taken as a standard for the preparation of AA solution. The AA content of the eggs used in the experiment was calculated on the basis of egg weight. The required amount of 20 crystalline L-amino acids were weighed and dissolved in sterile distilled water. The pH of the solution was adjusted to 7.0 by the addition of NaOH solution, nullified for microbial count and kept at 4°C. Prior to *in ovo* injection the solution was preheated to 30°C.

Experiment 2

In ovo injection of critical AA : In order to evaluate the effect of supplementing limiting AA(s) on the developing embryo, different combinations of essential and non-essential AA were injected into the eggs at 14 d of embryonic age. The combination AA for the *in ovo* injections was based on the critical requirement of those AA during embryonic and post-hatch period and AA interrelationship. Fertile eggs were collected from a synthetic dam line females (broiler strain), which received the AA balanced diet. The eggs (n=300) were weighed, distributed into six groups and the groups were randomly placed in a forced draft incubator. On 14th d of incubation, five groups of eggs were injected with 0.5 ml of the respective AA solution (Table 1) through the pinhole with 11 mm needle at the narrow end of the egg under laminar flow. Other procedures were similar as described earlier. One control group, which did not receive any injection, was also maintained.

Birds and housing : The chicks hatched from a particular treatment were distributed in 4 tier battery brooder cages having thermostatic control of temperature with provisions for separate feeder, waterer and droppings trays. The battery brooders were kept in a well lighted and ventilated open sided house. The brooder temperature during 1st to 4th week was 35, 32, 29 and 26°C, respectively. The mean ambient house temperature during the study ranged between 17 to 22°C. Each treatment group had three replicates of 8-10 birds/replicate depending upon the hatch size, assigned randomly and reared upto 4 weeks-of-age. They were provided a broiler starter ration (230 g/kg CP and 12.55 MJ/kg ME). Management practices like vaccination at day old (Newcastle Disease, F1 strain and Infectious Bursal Disease) feeding, watering, lighting etc. were followed in all the test groups throughout the experiment. Growth performance, immune response, and development of immune and digestive organs were studied during growing period.

Development of immune and digestive organs : Growth of immune and digestive organs was recorded at 21 d of age. Four birds from each treatment were subjected to feed withdrawal overnight prior to slaughter the birds were killed by cutting the jugular vein and carotid artery on one side of the neck near the atlanto-occipital joint. After bleeding the carcasses were scalded at 58°C for 2 minutes, defeathered in a rotary drum picker and manually eviscerated. The weight and length of the organs were taken

Table 2. Deposition of Indian ink at different embryonic age, injection site and needle length

Embryonic age	Injection site	Needle length (mm)	Site of deposition
7th d	Broad end	24	Yolk sac of embryo
	Broad end	11	Air cell of egg
	Narrow end	24	Yolk sac of embryo
	Narrow end	11	Albumin of egg
14th d	Broad end	24	Yolk sac of embryo
	Broad end	11	Air cell and chorio-allantoic cavity of egg
	Narrow end	24	Yolk sac of embryo
	Narrow end	11	Albumin of egg

immediately. The bursa, spleen and upper three lobes of the thymus from the left side of the neck were dissected out, weighed and expressed as mg/100 g of live body weight.

The weight of the digestive organs i.e empty proventriculus, empty gizzard, liver (without gall bladder), pancreas (detached from the duodenal wall), length and weight of small intestine (duodenum, jejunum and ileum), large intestine and caeca were recorded and expressed as g/100 g live weight.

Immune response : i) Preparation of sheep red blood cell suspension : Blood was collected in Alsever's solution from the jugular vein of healthy sheep and centrifuged at 2,500 revolution per minute (rpm) for about 10 minutes. The supernatant was discarded and the red blood cells were washed thrice in phosphate buffer saline (PBS). Suspension of Sheep Red Blood Corpuscle (SRBC) 1% v/v in PBS was prepared and stored in refrigerator at 4°C until its use.

ii) Immunization and harvesting of immune serum : On 22 d post-hatch a 1.0 ml suspension of SRBC was injected intravenously to ten-birds in each treatment to study the primary antibody response to SRBC. After 5 days post-immunization, a 2 ml blood was collected from the wing vein by a 2 ml disposable syringe and immediately transferred to a 10 ml test tube. The blood was allowed to clot, the serum was collected, and frozen (-70°C) until analyzed for the antibody titers to SRBC.

Haemagglutination (HA) test

The antibody titre was determined by HA methods (Vander Zijpp et al., 1983; Siegel and Gross, 1980). PBS (50 µl) was distributed in each well of the micro titre plate and then 50 µl of serum was added in the first well. Two fold serial dilutions were made up to row 11 while row 12 was kept as the control. Fifty µl of 1% SRBC was added in each well and mixed by gentle tapping. The plates were covered and then incubated at 37°C for 1 h. Plates were read under bright light and the reciprocal of highest dilution showing clear agglutination was the end titre. The titers were expressed as log 2.

Cell mediated immunity

The cellular immune response was assessed by cutaneous basophilic hypersensitivity test *in vivo* by using Phytohaemagglutinin, lectin from *Phaseolus vulgaris* (PHA-P). At 22 d post-hatch, another ten birds from each treatment were selected and the toe thickness of both the left and right foot at 3rd and 4th interdigital space was measured by micrometer (AMES, Model No. A/25, USA accuracy±0.01 mm). Immediately after measurement 100 µg of PHA-P (suspended in 0.1 ml of PBS) and 0.1 ml of PBS was injected into right and left foot (control), respectively. The web swelling of both the feet were measured 24 h after injection. The response was determined by subtracting the skin thickness (mm) of first measurement from the second and the values of left foot (control) from the right foot (Corrier and DeLoach, 1990).

Data obtained from the above experiments were subjected to statistical analysis (factorial, completely randomized design} as per standard procedure (Snedecor and Cochran, 1980) and Duncan multiple range test (Duncan, 1955) for treatment effects. The least square analysis of variance technique as per Harvey (1975) was used for analysis of those data with unequal number of observations.

RESULTS

Standardization of *in ovo* injection

After injecting the Indian ink the eggs were broken and the deposition site was examined visually (Table 2). When eggs were injected with 24 mm gauge needle in the broad end, either on 7 or 14 d of incubation, in most cases the ink was deposited in the yolk sac of the embryo. In contrast when a 11 mm gauge needle was used for injection in the broad end, either on 7 or 14 d of incubation the ink deposited partly in the air cell and chorio-allantoic cavity. When eggs were injected with a 24 mm gauge needle in the narrow end, the ink was deposited in the yolk sac and appeared to injure the embryo. When eggs were injected at the narrow end of the egg with 11 mm gauge needle, the ink was deposited in the albumen of the egg with minimum contact to embryo.

There was a significant ($p<0.016$) difference in the hatchability of eggs when AA were injected at different sites with different gauge needle (Table 3). Hatchability was poor when AA were injected at broad end with 11 mm gauge needle or at narrow end with a 24 mm gauge needle.

Table 3. Effect of site of injection and needle length at 7th and 14th d of incubation on the hatchability and day-old chick weight

Attributes	Egg weight	Chick weight	Chick wt. to egg wt. ratio	Hatchability (%)
Un-injected control	61.22±0.95	42.38±0.69	69.24±0.41	85.0
7th d of incubation				
Broad end 24 mm gauge needle	59.33±2.33	41.40±2.08	69.62±1.16	30.0
Broad end 11 mm gauge needle	59.96±1.85	41.62±1.53	69.38±0.65	10.0
Narrow end 24 mm gauge needle	60.46±2.08	41.64±1.95	68.75±0.93	20.0
Narrow end 11 mm gauge needle	60.78±1.73	41.58±1.59	68.33±0.87	65.0
14th d of incubation				
Broad end 24 mm gauge needle	60.04±2.42	42.36±1.87	70.47±0.64	85.0
Broad end 11 mm gauge needle	60.70±0.43	42.97±0.58	70.78±0.45	10.0
Narrow end 24 mm gauge needle	60.53±0.29	42.63±0.32	70.43±0.23	15.0
Narrow end 11 mm gauge needle	60.59±1.38	43.24±1.05	71.38±0.80	90.0
Main effects				
7th d	60.09±0.98	41.55±0.87	69.04±0.47^{X}	31.3±12.0
14th d	60.32±1.25	42.71±0.97	70.76±0.39^{Y}	50.0±21.7
Sig level	NS	NS	p<0.018	p<0.016
Broad end	59.92±1.30	42.07±1.04	70.11±0.43	33.8±17.7
Narrow end	60.60±0.78	42.34±0.70	69.82±0.50	47.5±18.0
Sig level	NS	NS	NS	p<0.016
24 mm gauge needle	59.99±1.31	42.03±1.05	69.96±0.45	37.8±16.1
11 mm gauge needle	60.51±0.76	42.38±0.66	70.01±0.48	43.8±20.1
P value	NS	NS	NS	NS
SEM	0.81	0.66	0.32	12.0

Means bearing similar or no alphabet do not vary significantly (p>0.05).

Table 4. Effect of injection of specific AA on the hatchability and chick to egg weight ratio

Treatments	Egg wt. (g)	Chick wt. (g)	Chick:egg wt.	Hatchability (%)
Un inj. control	63.6±0.93	43.7±0.71^{a}	68.7±0.47^{a}	86.3
Lys+arg	63.1±0.61	44.4±0.51^{ab}	70.5±0.52^{ab}	78.1
Lys+met+cys	64.8±0.86	45.4±0.86^{ab}	70.0±0.77^{ab}	72.0
Thr+gly+ser	64.7±1.60	45.9±1.5^{ab}	70.7±0.78^{b}	61.6
Ile+leu+val	64.6±1.18	46.5±1.20^{ab}	71.9±0.76b^{c}	87.5
Gly+pro	64.2±1.07	47.1±0.94^{b}	73.3±0.58^{c}	78.3
SEM	0.41	0.39	0.29	-
P value	NS	p<0.074	p<0.001	-

Means bearing similar superscripts in a column do not differ significantly (p>0.10).

Table 5. Effect of AA injection on the body weight gain (g) of chicks

Treatments	Ist wk B.Wt (g)	3rd wk B. Wt (g)	0-3 wk gain (g)
Un Inj. control	72.63±2.06^{a}	241.2±17.9	197.5±18.2
Lys+arg	74.73±2.17^{a}	264.0±20.0	219.6±19.9
Lys+met+cys	78.9±1.51^{ab}	273.6±14.6	228.1±14.7
Thr+gly+ser	81.6±5.62^{ab}	271.0±10.4	223.8±10.5
Ile+leu+val	87.8±1.12^{b}	304.8±29.2	256.6±30.1
Gly+pro	78.8±0.94^{ab}	304.4±5.11	257.9±5.2
SEM	1.51	8.20	8.08
P value	p<0.028	NS	NS

Means bearing similar superscripts in a column do not differ significantly (p>0.05).

However, better (p<0.016) hatchability was recorded when AA was injected at broad end with 24 mm gauge needle or at narrow end with 11 mm gauge needle. The chick weight to egg weight ratio was comparatively higher (p<0.018) when injected with 11 mm gauge needle at narrow end compared to eggs injected at broad end with 24 mm gauge needle.

In ovo injection of critical AA

Though, there was no difference (p>0.05) in the hatchability among the AA injected groups (61.6- 87.5% on the basis of fertile egg set), in some of the AA injected groups hatchability was lower than control group (86.3%). There was no difference in the egg weight of amino acid injected groups. However, chick weight (p<0.074) and the chick to egg weight ratio (p<0.001) were significantly higher in the AA injected groups, especially in Gly+Pro (Table 4). The data indicated that though Gly and Pro both are non-essential AA, the requirement for these two AA were very high during embryonic growth and differentiation.

Growth performance

The chick weight (p<0.07) and 7 d body weight was

Table 6. Effect of AA injections on feed intake and FCR of broiler bird during the growing period

Treatments	FI-1st (g/bird)	FI-3rd (g/bird)	FCR-1st	FCR 0-3 wk
Un inj. control	100.0±0.00[a]	329.3±9.3[a]	3.51±0.30	3.22±0.26
Lys+arg	98.0±1.15[a]	331.4±5.7[a]	3.27±0.30	3.11±0.14
Lys+met+cys	111.0±7.11[ab]	385.8±37.5[b]	3.32±0.07	3.00±0.24
Thr+gly+ser	105.6±6.9[a]	467.4±40.5[c]	3.15±0.30	3.40±0.33
Ile+leu+val	128.6±3.6[b]	505.2±46.9[c]	3.25±0.08	3.19±0.33
Gly+pro	101.9±11.3[a]	376.8±11.5[b]	3.00±0.48	2.74±0.07
SEM	3.31	19.05	0.11	0.10
P value	p<0.047	p<0.006	NS	NS

Means bearing similar superscripts in a column do not differ significantly (p>0.05).

Table 7. Immune response and weight of immune organs in specific AA injected birds

Treatments	Immune response[1]		Weight of immune organs[2] (mg/100 g live weight)		
	Cell mediated (PHA-P) toe web thickness (mm)	Humoral (SRBC) HA titre (log2)	Bursa	Thymus	Spleen
Control	0.220±0.02[a]	11.00±0.49[a]	81.30±17.2	115.5±18.75	174.5±18.0
Lys+arg	0.245±0.06[ab]	11.67±0.33[ab]	85.20±7.46	102.3±23.85	197.9±21.63
Lys+met+cys	0.379±0.04[bc]	12.43±0.30[abc]	105.0±38.40	121.5±14.68	243.9±50.21
Thr+gly+ser	0.412±0.04[bc]	14.00±0.84[c]	63.80±10.30	93.66±25.14	234.6±28.89
Ile+leu+val	0.349±0.08[bc]	13.50±0.71[bc]	98.80±16.40	104.4±26.09	198.1±28.58
Gly+pro	0.292±0.03[abc]	14.63±0.89[c]	121.0±57.2	115.4±19.94	205.2±20.53
SEM	0.02	0.31	11.6	8.11	11.88
P value	p<0.033	p<0.002	NS	NS	NS

[1] Mean±SE of 10 observations. [2] Mean±SE of 4 observations.
Means bearing similar superscripts in a column do not differ significantly (p>0.05).

Table 8. Effect of *in ovo* injection of specific amino acid on the digestive organ weight of broiler at 21 d of age

	Liver	Pancreas	Gizzard	Proventriculus	Small intestine length	Small intestine wt	Large intestine length	Large intestine wt	Caeca length	Caeca weight
Control	3.44±0.09	0.34±0.02	2.92±0.11	0.76±0.15	19.34±0.88	6.02±0.49	4.07±0.23	0.99±0.08	2.29±0.14	0.95±0.15
Lys+arg	3.39±0.19	0.36±0.02	2.89±0.01	0.68±0.05	21.23±1.25	6.58±0.25	3.71±0.18	1.04±0.10	2.12±0.20	1.07±0.08
Lys+met+cys	3.43±0.12	0.33±0.11	2.88±0.15	0.68±0.08	18.83±1.12	5.03±0.32	3.35±0.22	0.87±0.09	2.07±0.19	1.00±0.17
Thr+gly+ser	3.39±0.15	0.32±0.01	2.84±0.09	0.62±0.01	16.93±1.13	6.09±0.50	3.60±0.32	1.02±0.14	2.12±0.20	0.98±0.11
Ile+leu+val	3.48±0.28	0.29±0.01	2.83±0.12	0.65±0.04	18.27±1.20	5.40±0.43	3.54±0.50	0.98±0.08	2.11±0.10	1.10±0.17
Gly+pro	3.20±0.10	0.36±0.02	2.88±0.15	1.36±0.74	18.97±1.13	5.54±0.26	3.36±0.17	1.07±0.17	1.90±0.10	0.85±0.04
Sem	0.06	0.01	0.04	0.12	0.49	0.18	0.11	0.04	0.05	0.04
Sig. level	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

Means bearing similar superscripts in a column do not differ significantly (p>0.05).

significantly (p<0.028) higher in the AA injected groups (Table 5), especially in Thr+Gly+Ser, Ile+Leu+Val and Gly+Pro. At 21 d of age, Ile+Leu+Val and Gly+Pro group had 63.6 and 63.2 g higher weight gain, respectively than the un-injected control. Significantly higher (p<0.006) feed intake was recorded in the AA injected groups compared to the control. Though the FCR was not significantly different (p<0.11), a numerically better FCR was observed in the AA groups Gly+Pro, Lys+Met+Cys than in the control (Table 6).

Immune response

There was significant difference in the cell mediated (p<0.033) and humoral (p<0.002) immune response in *in ovo* AA injected birds, especially those injected with the combination of Lys+Met+Cys, Thr+Gly+Ser and Ile+Leu+Val. Though there was no significant difference in the weight of bursa and spleen but the weights were comparatively higher (p<0.16) in AA injected birds compared to control (Table 7).

Digestive organs

There was no significant difference in the weights of digestive organs and the development of intestine and caeca between AA injected groups and un-injected control during the early stages of growth (Table 8). The length of small intestine (p<0.068) and caeca (p<0.14) was comparatively lower in AA injected birds.

DISCUSSION

Injection site, needle length and embryonic age for *in ovo* injection

There is a good relationship between injection site and

needle length in *in ovo* injection of AA. AA deposited either in the air cell or in the chorio-allantoic cavity decreased hatchability, however, there was no adverse effect (Ohta et al., 2002) when injected into the yolk sac or the extra embryonic cavity. They have also reported that 13 mm gauge needle was suitable for *in ovo* injection in to the broader end of the egg. In our study it was revealed that when using a smaller needle (11 mm) injected in to the broad end, the deposition of AA was in the air cell or just below the air cell, which severely affected the survivability of embryo. When injected in to the narrow end, the hatchability was improved. It was also found that when using 24 mm gauge needle, AA injected in to the broad end on the 14th d of incubation did not affect the hatchability. In contrast to the our study Ohta and Kidd (2001) reported a significant decrease in hatchability when AA were injected in to the broad end with a 19 mm gauge needle as compared to 13 mm gauge needle on he 7th d of incubation. As found with the longer needle the deposition of AA was in the yolk sac in close proximity to the embryo. AA injected in to early stage (7th d) of incubation might be detrimental on chick growth and could be avoided by injecting on 14th d as demonstrated in our study.

Chick weight and hatchability in critical AA injected eggs

When injected with specific AA the hatchability was not affected. Instead in some of the AA injected groups, hatchability was higher than in the un-injected control. In the Thr, Gly and Ser group the hatchability was comparatively lower, because in those groups more deaths were recorded while chicks were piping the shell. Significantly higher chick weight at hatch in the Gly and Pro group was observed. Though these two amino acids are non-essential during post-hatch growth. Their requirement during incubation is higher. Ohta et al. (1999) reported that the proportion of Gly and Pro in the embryo increased during incubation. Pro is synthesized from several AA, where as precursors of Gly are limited to only Thr and Ser. Total molecular weight of Gly, Ser and Thr remained constant throughout the incubation period.

Growth performance in *in ovo* AA injected birds

When different combinations of specific amino acids were injected a difference in the early growth of birds (0-3 weeks) were noticeable in Ile+Leu+Val (63.6 g) and Gly+ Pro (63.2 g) AA injected groups compared to the un-injected control. Comparison of the AA composition of the egg and embryo at different stages of incubation also revealed that these AA are limiting for embryonic growth. In our earlier study (Bhanja, 2003) when all 20 AA were injected (0.5 ml of solution containing each AA at the concentration of 0.5% of that AA in egg) there was a 37 g higher weight gain during 0-3 weeks period. The body weight gain in specific AA injected chicks were comparatively higher than that obtained by Al-Murrani (1982). Proportion of Gly and Pro in embryo increases with incubation time and there was inter conversion of these amino acids to fulfill the requirement of the developing embryo (Ohta et al., 1999). Therefore, *in ovo* injection of Gly and Pro might have resulted in higher chick weight and subsequent post-hatch growth. However, the exact cause of higher chick growth in Ile+Leu+Val treatment group could not be established. Ohta et al. (1999) also reported that by 14th d of incubation the utilization of Ile, Leu and Val in proportion to pre-incubated content was comparatively higher than other AA. Injection of those amino acids might have stimulated higher protein synthesis and lower protein degradation. Venerando et al. (1994) reported that free AA in the diets of rats also decreased the rate of protein degradation.

Injection of specific AA significantly increased the feed consumption, which was also reflected in FCR of those birds. In our previous study (Bhanja et al., 2003) *in ovo* injection of all 20 AA resulted numerically higher feed consumption. Thr is closely associated with the digestive enzymes and mucous in the digestive tract of chicken. Stoll et al. (1998) reported that Thr retained by intestine and used for mucin formation or catabolized by enterocytes. Similarly, Bertolo et al. (1998) found that in 3 d pigs 45% of dietary Thr requirement was needed to support the activity of digestive enzymes in the intestinal mucosa. However, *in ovo* injection of Thr in combination with Gly and Ser in the present study did not affect the growth of digestive organs to support the higher secretion of mucous and other digestive enzymes as reported by previous workers.

Immune response in *in ovo* injected chicks

In the present study *in ovo* injection of Lys and Arg did not affect the growth of immune organs and response to SRBC or PHA-P. Lotan et al. (1980) reported that Lys does not have significant effect on antibody titer in rats. Kidd et al. (2001) also reported that commercial broilers fed Arg levels above NRC (1994) recommendations did not increase the weight of immune organ, primary antibody response to SRBC and cutaneous basophilic hypersensitivity responses (cell mediated immunity). Whereas, *in ovo* injection of Lys, Met and Cys had a significant effect on response to SRBC and PHA-P. This observation was in line with the findings of Tsiagbe et al. (1987). They reported that Met supplementation resulted in significant dose related increase in total antibody, IgG and response to PHA-P, but not in IgM. They suggested that Met was required for some components of the antibody response and might be required for thymus derived (T) cell helper function. Swain and Johri

(2000) also found that Met supplementation has higher leucocyte migration inhibition value and enhanced antibody titer of Newcastle Disease (ND) virus.

Konashi et al. (2000) reported that the branch chain amino acids (Ile, Leu and Val) affect the humoral and cell mediated immune response by affecting antibody production. It supported our finding as *in ovo* injection of Thr+Gly+Ser and Ile+Leu+Val resulted in higher antibody titer against SRBC and response to PHA-P. An analysis of the AA composition of immunoglobulin, found that Thr, Leu and Val content was comparatively higher in immunoglobulin (Tenenhouse and Deutsch, 1966). Higher production of antibody in those groups might have been due to higher supply of corresponding substrate through *in ovo* injection. This also supported the findings of Bhargav et al. (1970,1971). They observed that a deficiency of Val and Thr reduced the antibody production against ND virus in chicken.

Enteric development in amino acid injected birds

In the present study there was no significant difference in the development of the digestive organ and intestine at 21 d of age. Coles et al. (2001) reported that *in ovo* administration of 600 μg pancreatic poly peptide (PYY)/kg egg weight in broiler chicken and turkey eggs resulted in increased body weight and feed efficiency of chicks and poults during the first week post-hatch. They postulated that PYY enhanced the absorptive capacity of hatchlings, which resulted in enhanced growth. Ferket and Uni (2002) also reported that *in ovo* injection of 1 ml of saline containing carbohydrate at the 18th d of incubation resulted in significant increase in jejunum villi height by over 45% after 48 h. In the present study the length of small intestine and caeca was comparatively lower in AA injected birds. Mandal et al. (2004) reported decrease in the length of small and large intestine in Met supplemented diet.

CONCLUSION

It may be concluded that Thr, Gly, Ser and branched chain amino acids (Ile, Leu and Val) were critical for the growth of chicken embryo. *In ovo* injected amino acids may also act as immunomodulatory and their role in gastrointestinal development needs further research. This preliminary study may open a window for future research in to this aspect.

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