

# Differential expression of growth and immunity related genes influenced by *in ovo* supplementation of amino acids in broiler chickens

S.K. BHANJA, M. SUDHAGAR, A. GOEL, N. PANDEY, M. MEHRA, S.K. AGARWAL, A. MANDAL

Central Avian Research Institute, Izatnagar, India

**ABSTRACT:** The present study was aimed at investigating the role of *in ovo* administered amino acids: lysine, arginine, threonine or methionine plus cysteine (Met+Cys) in 14-day embryos on expression profile of growth (chicken growth hormone (*cGH*), insulin like growth factors (*IGF*) I and II, and *mucin*) and immunity related genes (*IL-2*, *IL-4*, *IL-6*, *IL-12*, *TNF- $\alpha$* , and *IFN- $\gamma$* ). On incubation day (ID) 18, higher ( $P < 0.01$ ) *cGH* and *mucin* gene expression was observed in lysine, threonine, arginine or Met+Cys injected embryos, while *IGF-II* expression was higher in threonine, arginine or Met+Cys injected embryos on ID 20. Expression of growth genes was down regulated ( $P < 0.01$ ) on day of hatch in most of the amino acids injected chicks. On day 7 post-hatch (PH), threonine or arginine exhibited higher expression of *cGH*, *IGF-I*, and *IGF-II* but higher *mucin* gene expression only on day 14 PH. Threonine or Met+Cys injected birds had higher expression of *IL-6* and *TNF- $\alpha$* , while arginine injected birds had higher *TNF- $\alpha$*  expression. Lysine, threonine or Met+Cys injected birds had higher *IL-2*, but lower of *IL-12* and *IFN- $\gamma$*  gene expression. It is concluded that arginine and threonine enhanced the expression of growth related genes, while threonine and Met+Cys modulated expression of immune genes in broiler chickens.

**Keywords:** *in ovo* injection; critical nutrients; growth genes; immunity genes; broilers

## INTRODUCTION

Nutritional and hormonal factors are the major determinants of animal growth during fetal and post-natal development. The dietary intrinsic factors and related metabolic interactions have direct as well as indirect influence on growth via expression and regulation of genes. Moreover, the genes present on the somatotrophic axis respond to nutrition differently at the level of transcription (Beccavin et al. 2001). The growth hormone (GH) that regulates the growth exerts its effects mainly by insulin-like growth factor-I (IGF-I), which is synthesized in the liver under GH control and secreted into the circulation (Mc Murtry et

al. 1997). Protein or energy deficiency leading to reduced growth velocity was associated with lowered levels of plasma IGF-I (Straus and Takemoto 1990, 1991). Whereas dietary supplementation of a high protein diet with arginine, methionine, and cysteine enhanced plasma IGF-I levels and body weight gain in young chicks (Kita et al. 2002). Thus the early accessibility to critical dietary nutrients would be beneficial, but it depends upon the initiation of food consumption. In contrast, in the case of feeding the young birds with critical nutrients by *in ovo* injection, the initiation of the feed intake does not depend on the bird. Previous studies also suggested that *in ovo* supplementation of amino acids significantly increased growth in broiler

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chicks (Bhanja et al. 2004b; Bhanja and Mandal 2005; Kadam et al. 2008; Bakyaraj et al. 2012).

The development of mucus secreting cells in small intestine of chickens occurs during late embryonic age and immediately after post-hatch. Mucin is the main constituent of the mucus layer which can influence nutrient digestion and absorption. Dietary components have the potential to induce changes in mucin dynamics. The presence of nutrient in gastrointestinal tract (GIT) is also crucial for mucosal development and feed deprivation for the first 48 h post hatch (PH) resulted in delayed development of the mucosal layer and mucin synthesis in young chicks (Uni et al. 2003). Even the intra-amnionic nutrient administration accelerated small intestine development and had an enhanced effect on the function of enterocytes (Tako et al. 2004; Uni and Fekret 2004). In a recent work, it had been reported that Thr deficient diet reduced *MUC2* gene expression in ducklings that caused changes in mucin secretion affecting intestinal nutrition and absorption (Horn et al. 2009).

Besides growth, nutrients can also play an important role in functioning of the immune system. Upon activation, the naive T cells (Th0) differentiate into either T helper 1 (Th1) cells primarily responsible for cell mediated immunity and inflammation, or Th2 effector cells, eliciting humoral immunity by secreting different cytokines which mediate very different kind of immune responses (Spellberg and Edwards 2001; Staeheli et al. 2001).

Few studies have been conducted to evaluate the effect of *in ovo* amino acid administration on the PH performance but scanty information is available on the role of these nutrients on the expression profile of growth (chicken *GH*, *IGF-I* and *II*, and *mucin*) and immunity related genes (*IL-2*, *IL-4*, *IL-6*, *IL-12*, *IFN- $\gamma$* , and *TNF- $\alpha$* ) in broiler chickens. Emphasis is now laid on investigating the role of nutrients in growth process at molecular level. In addition, it will be interesting to see whether the nutrients would be able to modulate the expression of cytokines responsible for humoral or cellular immunity in chickens. Keeping in view the above facts, an effort was made to assess the effect of *in ovo* injection of critical amino acids (lysine: Lys, threonine: Thr, arginine: Arg, methionine and cysteine: Met+Cys) on the expression profile of growth and immunity related genes during late embryonic and early PH period. Result of the present study will help in precisising the diet for better growth and immune responsiveness of neonatal chicks.

## MATERIAL AND METHODS

**Experimental material and treatments.** The study was conducted using crystalline amino acids: Lys (55 mg), Thr (40 mg), Arg (62.5 mg), and Met+Cys (45 mg at 1 : 1 ratio) as *in ovo* supplement. Fertile eggs ( $n = 500$ ) were collected from broiler breeder flocks maintained on balanced ration as per National Research Council recommendations (NRC 1994) and were distributed into five groups (four amino acids and one uninjected control) of 100 eggs each. In our earlier study on *in ovo* feeding (Bakyaraj et al. 2012) no statistical variation was observed in the response of uninjected control and sham control (only placebo with no nutrients), so to reduce the sampling size we had excluded sham control from the present experimental design. After collection, the eggs were fumigated with formaldehyde gas (at 1X concentration) and incubated in a forced draft incubator at temperature of 37.5°C with relative humidity of 60%.

***In ovo* amino acid injection.** On incubation day (ID) 14, 0.5 ml of warm (30°C) amino acid solution was injected into the amniotic cavity through a pinhole made at the broad end of the egg, using a 24G hypodermic needle (25 mm long) following the technique of Bhanja et al. (2004a). The whole injection procedure was carried out under laminar flow system and the injection site was sealed with sterile paraffin wax. Immediately after injection the eggs were returned to the incubator and on ID 19, the eggs were transferred to the hatcher and placed in pedigree-hatching boxes. The hatched chicks were reared in electrically heated battery brooders and provided standard diets and management till 28 days of age.

**Tissue collection for growth related gene expression studies.** Four embryos or birds from each treatment group were killed through cervical dislocation to collect 200 mg of liver (for chicken growth hormone (*cGH*), *IGF-I* and *IGF-II* gene expression) and intestine tissue (jejunum, for *mucin* gene expression) on ID 18 and 20, on day of hatch (DOH), and on days 7 and 14 PH. The tissue samples were then homogenized in an automated Kinematica™ Polytron™ homogenizer PT 1200 E (Thermo Fisher Scientific, Inc., Waltham, USA) and 50 mg of homogenized tissues were collected for total RNA isolation. For contingency uses, about 150 mg of tissue were immersed in 500  $\mu$ l of RNA stabilizing solution in 1.5 ml centrifuge microtubes. These samples were kept at 4°C over-

night and then transferred to a deep freezer and kept at  $-80^{\circ}\text{C}$  for further use.

**Collection of samples for immune related gene expression studies.** *Candidate genes for humoral immunity:* To quantify the candidate genes of humoral immunity (*IL-6*, *IL-10*, and *TNF- $\alpha$* ), four *in ovo* supplemented birds were challenged with 1 ml of 1% (V/V) sheep red blood cells (SRBC) on day 21 PH and blood samples were collected after 5 days post-injection. Approximately 1.5 ml of blood from each bird was mixed with 3 ml of PBS (1 : 2 dilutions). An amount of 3 ml of Histo-paque<sup>®</sup> 1.077 (Sigma Diagnostics Inc., St. Louis, USA) was taken in a 15 ml conical centrifuge tube and the diluted blood was carefully layered over the column and centrifuged at 1800 *g* for 20 min at room temperature. After centrifugation the opaque interface containing peripheral blood mononuclear cells (PBMC) was carefully transferred to a clean conical centrifuge tube. 10 ml of isotonic PBS solution was added, mixed by gentle aspiration, and then centrifuged at 2500 *g* for 10 min. After centrifugation, the pellet was dissolved in a denaturing solution for total RNA isolation.

*Candidate genes for cell-mediated immunity:* For quantification of cellular immune related genes (*IL-2*, *IL-12*, and *IFN- $\gamma$* ), approximately 1.5 ml of blood was collected from the jugular vein of another four birds per group on day 21 PH and the PBMC cells were separated (as detailed earlier). Viability of these cells were determined by Trypan blue (0.4%) staining methods. Cells were resuspended in the known volume of RPMI-1640 (Sigma Diagnostics Inc.) to make the final concentration of 106 cells per ml of medium. PBMCs were plated in 6 well culture plates with RPMI-1640 (without phenol red) medium, supplemented with 10% FBS, 2mM L-glutamine, 2mM L-arginine under 5%  $\text{CO}_2$  tension in humidified atmosphere. The PBMC cells were sensitized to 10  $\mu\text{g}/\text{ml}$  Concanavalin A for a period of 4 h, then the PBMCs adhered to culture plates were harvested into a 1.5 ml Eppendorf tube (Eppendorf, Hamburg, Germany), and centrifuged at 5000 *g* for 1 min. After centrifugation, the pellet was dissolved in denaturing solution for total RNA isolation.

**RNA isolation and reverse transcription.** Total cellular RNA from liver and jejunum of each treatment group was isolated using RNeasy<sup>®</sup> Total RNA Isolation System (Promega, Madison, USA), purity and quantity were assessed by measuring the optical density of each sample at 260 versus 280 nm in a nanodrop. Any possible traces of

genomic DNA were removed by treating 5  $\mu\text{g}$  of each RNA sample with 5 U of RNase-Free DNase (Biogen Idec, Inc., Durham, USA) at  $37^{\circ}\text{C}$  for 1 h. The DNase was subsequently inactivated by incubation at  $65^{\circ}\text{C}$  for 10 min. Each DNase treated total RNA sample (2  $\mu\text{g}$ ) was reverse transcribed using the RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas, Hanover, USA) according to the manufacturer's instructions. The reverse transcription reaction was carried out in a final volume of 20  $\mu\text{l}$ . The resultant cDNA was stored at  $-20^{\circ}\text{C}$  for further use.

**Standardization of primers for PCR.** The primers for this study were designed using DNASTAR Lasergene software (Version 5.0, 1997). The specificity of primers was checked by the NCBI blast program. The qPCR assays were evaluated by the generation of a standard curve. Calibration curves for each gene were done on an iQ5 cycler (Bio-Rad Laboratories, Hercules, USA) with five 10-fold serial dilutions (in triplicates) and were calculated by the Bio-Rad Optical System Software (Version 2.1, 2010) with the analysis mode "PCR base line subtracted". Amplification efficiency (E) of qPCR reactions was calculated with the slope of the log-linear portion of the calibration curve according to the equation:  $E = 10^{-1/\text{slope}}$  (Rasmussen 2001; Bustin et al. 2009). PCR products for different genes were sequenced and the sequences were submitted to NCBI, USA or EMBL, UK for confirmation. The genes with the assigned accession numbers are reported in Table 1.

**Expression of growth and immune related genes by real time PCR.** The amplifications of growth and immune related genes were carried out using an iQ5 cycler (Bio-Rad Laboratories) in 25  $\mu\text{l}$  volume containing 1X QuantiTect<sup>®</sup> SYBR<sup>®</sup> Green PCR Master Mix (SYBR<sup>®</sup> Green 1 dye, HotStartTaq DNA polymerase, and dNTPs in optimized buffer components; Qiagen GmbH, Hilden, Germany), a 0.2 $\mu\text{M}$  concentration of each gene-specific primer, and 1  $\mu\text{l}$  of cDNA template. PCR cycling conditions included initial denaturation at  $95^{\circ}\text{C}$  for 10 min, followed by 40 cycles of denaturation at  $95^{\circ}$  for 30 s, annealing for 30 s, and extension at  $72^{\circ}$  for 45 s. For each gene of interest, negative and positive controls were also included. Negative controls were samples in which cDNA was not added. A melting curve was performed for each sample after completion of amplification and analyzed in comparison to negative and positive controls to determine the specificity of PCR reaction.

Table 1. Oligonucleotide sequence of growth and immune related gene primers

Gene <sup>1</sup>	Sequence (5' → 3')	Annealing temperature (°C)	Product size (bp)	Accession number	E* (%)
<b>Major growth related genes</b>					
<i>cGH</i>	F-CACCACAGCTAGAGACCCACATC R-CCCACCGGCTCAAACCTGC	58	201	HE608816	90
<i>IGF-I</i>	F-GGTGCTGAGCTGGTTGATGC R-CGTACAGAGCGTGCAGATTTAGGT	58	203	JN942578	96
<i>IGF-II</i>	F-GGCGGCAGGCACCATCA R-CCCAGCAGCAAAAAGTTCAAAG	58	215	JN942579	100
<b>Intestinal tract development related gene</b>					
<i>Muc</i>	F-CTGGCTCCTTGTGGCTCCTC R-AGCTGCATGACTGGAGACAACCTG	58	242	JN639849	92
<b>Humoral immunity related genes</b>					
<i>IL-4</i>	F-AATGACATCCAGGGAGAGGTTTC R-GCTAGTTGGTGGAAGAAGGTACG	55	219	JN639847	86
<i>IL-6</i>	F-GAAATCCCTCCTCGCCAATCTGA R-TGAAACGGAACAACACTGCCATCT	57	281	NM001007079	96
<i>TNF<math>\alpha</math></i>	F-AGACCAGATGGGAAGGGAATGAA R-GAAGAGGCCACCACACGACAG	55	219	JN942589	91
<b>Cell mediated immunity related genes</b>					
<i>IL-2</i>	F-CCCGTGGCTAACTAATCTGCTG R-TGAGACACCAGTGGGAAACAGT	57	287	HE608819	89
<i>IL-12</i>	F-GCCGACTGAGATGTTCTCTGG R-CCTTGCTTTTGTATTTCTTTGTGC	57	227	JN942590	92
<i>IFN<math>\gamma</math></i>	F-AGCTGACGGTGGACCTATTATTGT R-CGGCTTTGCGCTGGATTC	58	260	JN942588	86
<b>Reference gene</b>					
<i>28S</i>	F-CAGGTGCAGATCTTGGTGGTAGTA R- GCTCCCGCTGGCTTCTCC	58	273	JN639848	98

<sup>1</sup>*cGH* = chicken growth hormone, *IGF* = insulin-like growth factor, *Muc* = mucin, *IL* = interleukin, *TNF $\alpha$*  = tumor necrosis factor alpha, *IFN $\gamma$*  = interferon gamma, *28S* = 28S rRNA

\*efficiency (E) was calculated by the slope of the standard curve by the equation:  $E = 10^{-1/\text{slope}}$

The relative expression ratio (ER) of a target gene is computed, based on its real-time PCR efficiencies (E) or a static efficiency of 2, and the cycle threshold (*Ct*) difference ( $\Delta$ ) of mean control versus each unknown sample ( $\Delta C_t$  control – treatment) as described below (Pfaffl 2001) using 28S rRNA as the reference housekeeping gene:

$$ER = \frac{(E \text{ target})^{\Delta C_t \text{ target (control - treatment)}}}{(E \text{ ref})^{\Delta C_t \text{ ref (control - treatment)}}$$

**Statistical analysis.** mRNA expression levels (ER) of growth related and immune related genes were analyzed by One Way ANOVA using the SPSS

software package (Version 16.0, 2007). Difference in mean values was considered as significant at the level of 95% ( $P < 0.05$ ) and 99% ( $P < 0.01$ ).

## RESULTS

**Expression pattern of growth related genes.** During pre-hatch (ID 18 or 20), the relative expression of hepatic *cGH* gene was increased in all the amino acid (Lys, Thr, Arg, and Met+Cys) injected embryos compared to uninjected control. While on DOH, hepatic *cGH* expression decreased ( $P < 0.01$ ) in Lys or Thr treated embryos than in the uninjected control. During PH on day 7, Thr or

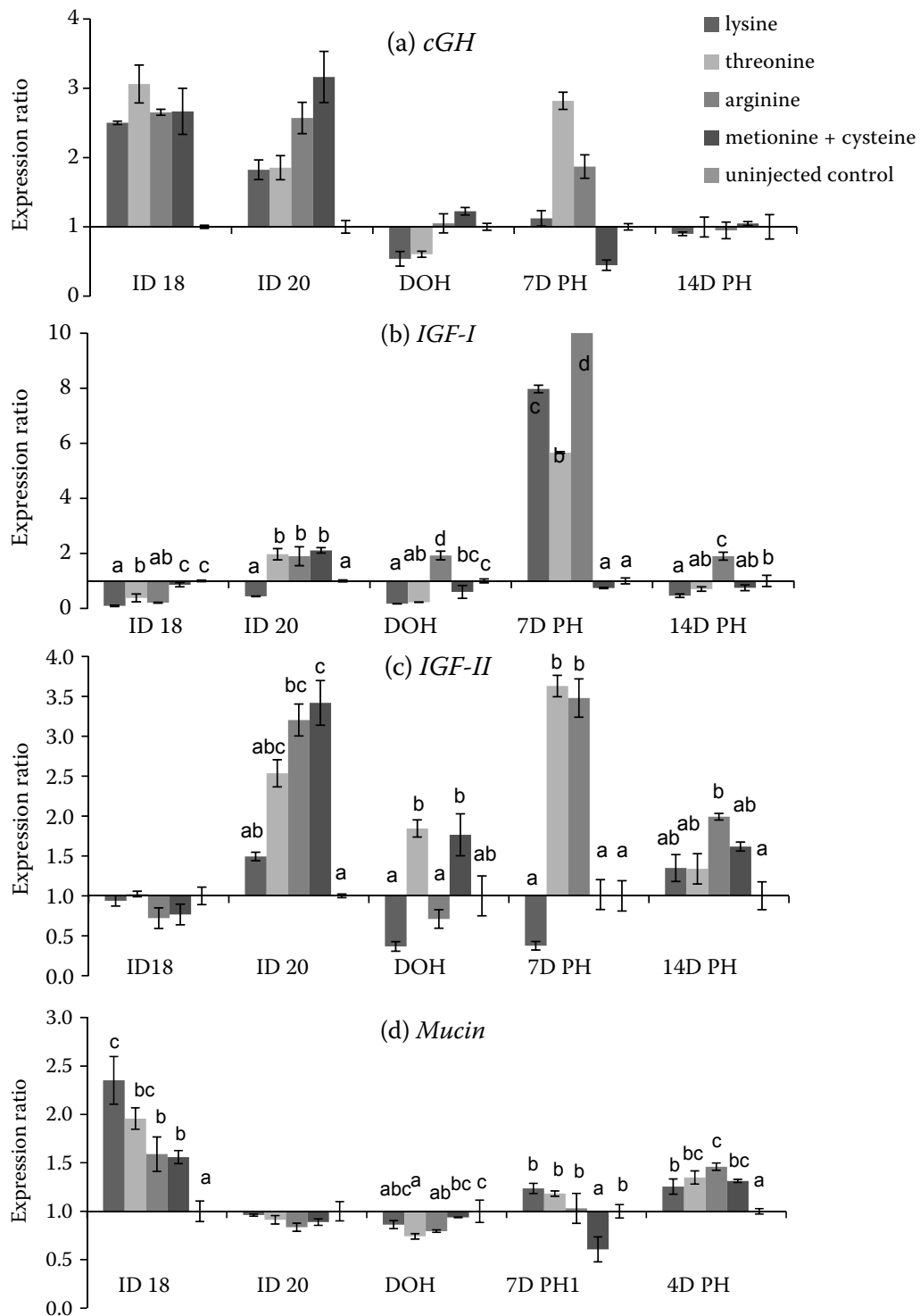


Figure 1. Relative expression of hepatic *cGH*, *IGF-I*, *IGF-II*, and intestinal *mucin* genes during late embryonic and early post-hatch period. Expression ratio is the mean of four observations

ID = incubation day, DOH = day of hatch, D = day, PH = post hatch

<sup>a-d</sup>significant differences ( $P < 0.05$ )

Arg injected embryos had two fold higher hepatic *cGH* expression ( $P < 0.01$ ) but on day 14 the expression was similar in amino acid (AA) injected and uninjected control (Figure 1a).

During pre-hatch on ID 18 hepatic *IGF-I* gene expression decreased ( $P < 0.05$ ) in Lys, Thr or Arg injected chicks, but it increased on ID 20. During PH, the expression increased ( $P < 0.01$ ) in all the

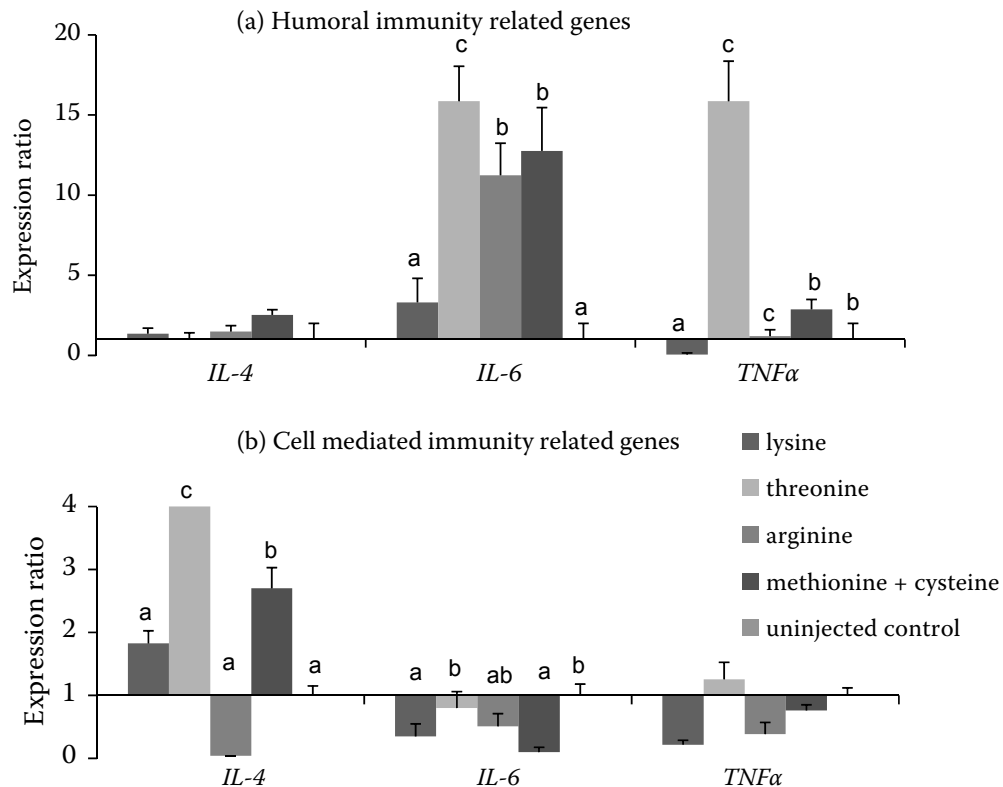


Figure 2. Expression ratio of humoral (*IL-4*, *IL-6*, *TNFα*) and cell mediated immunity (*IL-2*, *IL-12*, *IFN-γ*) related genes. Values are mean of four observations

<sup>a-c</sup>significant differences ( $P < 0.05$ )

Arg injected chicks and in Lys or Thr injected chicks on day 7 PH when compared to uninjected control (Figure 1b).

Expression of hepatic *IGF-II* gene did not differ in AA injected embryos on ID 18 but on ID 20 a higher ( $P < 0.01$ ) expression was observed in Arg or Met+Cys treated embryos. During PH the expression was higher in Thr or Arg injected chicks on day 7 PH ( $P < 0.01$ ) and in Arg injected chicks on day 14 PH ( $P < 0.05$ ) than in uninjected control chicks (Figure 1c).

On ID 18 and day 14 PH, higher ( $P < 0.01$ ) intestinal *mucin* gene expression was observed in Lys, Thr, Arg or Met+Cys injected embryos or chicks, while no such differences were observed on ID 20 or DOH. But on day 7 PH, the expression decreased ( $P < 0.01$ ) in Met+Cys chicks compared to uninjected control (Figure 1d).

**Expression pattern of immune related genes.** No variation was observed in the expression of *IL-4* gene in AA injected or control chicks, however, the expression of *IL-6* gene was increased ( $P < 0.01$ ) in Thr, Arg or Met+Cys injected chicks compared to uninjected control group. The ex-

pression of *TNF-α* gene was up regulated in Thr or Arg treated chicks but down regulated in Lys treated chicks (Figure 2a).

Expression of *IL-2* gene was up regulated in Thr or Met+Cys treated chicks, while no variation was observed in Lys or Arg injected chicks in comparison to uninjected control group. The expression of *IL-12* gene was decreased ( $P < 0.05$ ) in Lys or Met+Cys while no difference was observed in the expression of *IFN-γ* gene in AA injected chicks when compared to uninjected control group (Figure 2b).

## DISCUSSION

Growth hormone (GH) is necessary for differentiation of muscle, adipocytes, and other cells to modulate development and growth (Kim 2010). In the present study, increased expression of hepatic *cGH* gene was observed in AA injected embryos during pre-hatch period, indicating an effective way to increase the size of the chick without decreasing hatchability (Ohta et al. 1999). The highest growth rate in domestic chicken occurs during

the late embryonic to juvenile stages of development (Kuhn et al. 2002), and injection of the critical AA (Lys, Thr, Arg or Met+Cys) might have helped the developing embryo to overcome the nutritional stress, where yolk is the only source. These results also correlated with the earlier studies where increase in body weight of broilers was reported when GH was administered *in ovo* at specific days of embryogenesis (Kocamis et al. 1999). During PH, growth hormone can be very useful for the normal development of the embryo (King and Scanes 1986), which was quite evident from the present study as significant increase in the expression of *cGH* gene was observed in Thr or Arg treated chicks. Earlier findings also suggested that *in ovo* administration of all 20 AA increased chick weight by 2.1–3.6% (Ohta et al. 2001; Bhanja et al. 2004b). Even *in ovo* injection of specific AA (Lys+Arg, Lys+Met+Cys or Thr+Gly+Ser) induced by around 2.0% higher chick weight at hatch and by 10–13% higher body weight at day 21 PH (Bhanja and Mandal 2005). In a recent study also higher PH body weight was observed in *in ovo* Arg treated chicks (Bhanja et al. 2012).

It is suggested that IGF-I synthesis is GH-independent during embryogenesis (Tanaka et al. 1996). This was also evident on ID 20, when both *IGF-I* and *cGH* gene expression increased in Thr, Arg, and Met+Cys treated chicks that might have helped the embryos to achieve higher skeletal muscle growth by reducing protein degradation and/or by increasing protein synthesis (Tomas et al. 1998; Conlon and Kita 2002). Generally, IGF-I and -II are responsible for proliferation of pre-adipocytes, chondrocytes, and fibroblasts through amino acid stimulation, glucose uptake, increased DNA synthesis, tissue growth stimulation, and overall embryogenesis regulation (McGuinness and Cogburn 1990; Leach and Rosselot 1992; Guernec et al. 2003). In the present study the expression of both *IGF-I* and *IGF-II* increased in Arg treated groups, too. Vasanti (2009) reported that relative *IGF-II* expression was higher in control group than in other treatments on DOH, but the expression increased with age where all PH supplemented groups showed higher relative *IGF-II* levels than control.

Previously, Uni et al. (2005) suggested that *in ovo* injection of Arg increased broiler hatchling weights, breast muscle, and improved hepatic glycogen reserves over the controls. Dietary supplementation of Arg enhanced plasma IGF-I levels

and body weight gain in young chicks (Kita et al. 2002). Similarly, there are reports that *cGH* mRNA expression and serum concentration were enhanced by Arg, biotin, and Lys supplementation (Kim et al. 1991, 2004). Another AA which also enhanced the *cGH* and *IGF-I* expression in our study was Thr, that has a specific regulatory role in the secretion of pancreatic enzymes (amylase, trypsinogen, and chymotrypsinogen) in chickens (Yang et al. 1989). Dietary Thr level had also a significant effect on the average daily gain and feed conversion ratio (Kadam et al. 2008). Moreover, Thr is a precursor for synthesis of the amino acid glycine required during production of uric acid in chickens.

Very few information is available on the effects of dietary nutrients on mucin synthesis in poultry species. A decreased mucin synthesis result of a compromised mucus layer could reduce nutrient utilization. It is also reported that *in ovo* carbohydrate injected embryos had greater villus surface area from day 19 of incubation until 3 days PH, higher goblet cell density of jejunum villi, and higher *mucin* gene expression than the saline-control birds (Smirnov et al. 2006). Certain amino acids such as Thr, Ser, and Cys are of particular interest because these are the key components in mucin amino acid backbone (Gum Jr.1992). The hydroxyl group of Thr and Ser is necessary for ester linkages to carbohydrate groups that make up the majority (50–80%) of the molecular weight of mucin (Montagne et al. 2004). When dietary Thr level increased, there was an increase in intestinal goblet cell density and *MUC2* expression (Horn et al. 2009). In the present study, significantly higher expression of *mucin* gene in Thr injected chicks over control during PH was observed. Further, higher expression of mucin gene was also seen in Arg or Met+Cys injected chicks, suggesting the presence of these amino acids might have triggered the number of goblet cells and production of acidic mucin in the GIT of a chick embryo. The expression of *mucin* gene is directly related with the development of small intestine. In a recent study, higher small intestinal weight in Lys, Thr, and Arg treatments but lower weight in Met was reported (Bhanja et al. 2012). These findings corroborated with our study wherein expression of *mucin* gene was increased in Lys, Thr, and Arg treatment while decreased significantly in Met+Cys treatment.

In the present study the expression of *IL-4*, *IL-6*, and *TNF- $\alpha$*  was higher in Met+Cys injected birds than in those of uninjected control and lysine

group, and reached a significant level only in *IL-6* (for Met+Cys) and *TNF- $\alpha$*  (for Lys). This is in line with the earlier report of Sirimongkolkasem (2007), who suggested that Met plays an important role in growth and humoral immunity (antibody production), and Cys is in greater demand than lysine for humoral immunity. Cysteine also added to the glutathione and accessory protein produced by liver during the acute phase response (Grimble 2006). In another study, both Coccidiosis and Newcastle Disease infections were severe in chicks fed methionine deficient diets (Cook, 1991). Thr treatment was found to increase the expression of humoral immune genes (*IL-4*, *IL-6*, and *TNF- $\alpha$* ), though *IL-4* expression did not reach a significant level. Higher humoral immune response could be attributed to higher Thr content in the immunoglobulins (Tenenhouse and Deutsch 1966).

In the present study the expression of *IL-2* gene increased in Thr or Met+Cys treatment, possibly related to the cell-mediated immunity (Kidd et al. 1996; Shyam Sunder et al. 2008). In earlier studies significant difference in cell mediated immunity was observed in *in ovo* injection of AA combinations containing Lys, Thr or Met+Cys (Bhanja and Mandal 2005; Bakyaraj et al. 2012) and they also increased the relative weight of spleen (Bhanja et al. 2012). The expression of *IL-12* gene decreased in all the amino acid injected birds (Lys, Thr, Arg, and Met+Cys) in comparison to uninjected control, but it did not reach the significant level in Thr and Arg treatments. The expression of *IFN- $\gamma$*  gene was reduced but insignificantly in most of amino acids except Arg in comparison to uninjected control chicks. There is also cross regulation between Th1 cells and Th2 cells (Spellberg and Edwards 2001) and it was also evident in the present study where expression of *IL-4* suppressed the expression of *IFN- $\gamma$*  and it is also reported that *IL-4* produced by Th2 cells block the differentiation of Th0 to Th1 (D'Andrea et al. 1993).

## CONCLUSION

Based on the above observation it was concluded that amino acids, particularly arginine and threonine, enhanced the expression of growth related genes (*cGH*, *IGF-I*, *IGF-II*, and *mucin* gene) during pre- and post-hatch periods. Moreover, threonine and sulphur containing amino acids (methionine plus cysteine) played a role in modulating immune gene expressions. There is a need of further re-

search on how these amino acids modulate at the protein level and the result of this study could be helpful in formulating the chick's diet for better growth and immunity.

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*Corresponding Author*

Dr. Subrat Kumar Bhanja, Principal Scientist, Central Avian Research Institute, Poultry Housing and Management Section, Izatnagar, 243 122 (UP), India  
Phone: +91 05 812 300 320, +919 359 105 979, e-mail: subratcari@gmail.com

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