DOES in ovo ADMINISTRATION OF ZINC OR IODINE MODULATE DIFFERENTIAL EXPRESSION OF GROWTH AND IMMUNE RELATED GENES IN BROILER CHICKENS

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ABSTRACT
In ovo administration of zinc (0.5mg) or iodine (17.5µg) was done in 14d incubated eggs for assessing the differential expression of growth and immunity related genes. Growth related genes, Insulin like growth factor-1 and 2 (IGF-1,2) were studied in hepatic tissue. Humoral immunity related genes: interlukin-6 (IL-6) and tumor necrosis factor (TNF-α) were studied in WBC’s of chicks following 5d post injection of sheep RBC’s, while expression of cellular immunity related genes: IL-2 and IL-12 were studied in PBMC culture after sensitizing with mitogen Con A. Total RNA was isolated by TRIZOL method and reverse transcription PCR (RT-PCR) was performed using 3-5 µg RNA. Amplification was done by semi-quantitative PCR and quantification of gene expression was done using Image J software and 28S rRNA as reference gene. IGF-1 expression was significantly higher in iodine injected chicks on day of hatch, while those having zinc injection had down regulation of IGF-1 during post-hatch period. IGF-2 expression was higher in both zinc and iodine injected embryos during late incubation and day of hatch, while during post-hatch only zinc injected chicks had higher expression in comparison to un-injected control. There was up regulation of IL-6 and TNF-α gene expression in iodine injected chicks but, down regulation in zinc injected chicks. Expression of IL-2 and IL-12 gene was significantly higher in zinc injected chicks. It is concluded that both zinc and iodine modulate growth related genes during late embryonic stage. Iodine plays a role in humoral immunity, while zinc has role in cellular immunity.

KEYWORDS: In ovo, Trace Mineral, Growth and Immunity genes

INTRODUCTION
Being a component of metalloenzymes, zinc is an important mineral for poultry. These metalloenzymes play important role in bird’s immune response, wound healing, and in hormone production (testosterone and corticosteroids). Similarly, iodine helps in growth and development of bird by stimulating TSH released from thyroid gland. Yolk is the major source of Zn and its consumption increases after E11 until E17 (Yair and Uni 2011). Recently, in ovo injection of nutrients directly in to the amniotic or yolk sac of embryo has been tried to improve chick size (Ohta et al., 1999) growth rate (Bhanja et al., 2004b) and immune response (Bhanja and mandal, 2005). Keeping this in mind an experiment was designed to study the role of in ovo injected zinc (Zn) and iodine (I) on the differential expression of growth and immunity related genes in broiler chickens.

MATERIALS AND METHODS
Fertile eggs (n = 300) were collected from broiler breeders flocks maintained at CARI Izzatnagar, and distributed in three groups of 100 each. On 14th day of incubation Zn (0.5mg)
and I (17.5µg) were injected into the yolk sac/amnion of the embryos, under laminar air flow system, following standard procedure of Bhanja et al. (2004a). Simultaneously one un-injected group was left as control. After hatching chicks were reared in electrically heated battery brooders kept in a well ventilated and lighted room and provided standard nutrition and management.

Liver samples (four/group) at different intervals i.e at 18th & 20th day of embryonic stage, on the day of hatch and on 3rd, 7th, 10th and 14th day post-hatch were processed for studying growth related genes (IGF-I and IGF-II). To study cellular immune related genes (IL-2 and IL-12) on 21st day post-hatch approximately 2 mL blood was collected from the jugular vein of 4 birds each per treatment and control group. Peripheral blood mononuclear cells (PBMC) were separated, cultured and sensitized with mitogen Con A (10µg/ml) and after 4 hrs the cells were harvested for total RNA isolation. Similarly to quantify humoral immune related genes (IL-6 and TNF–α) four birds from each group were challenged with 1 mL sheep red blood cells (SRBC) of 1% (V/V) on 14th day post-hatch and approximately 2 ml blood was collected on 5th day post-injection to isolate WBCs.

Total RNA Isolation was done by Trizol method (RNAgents, Promega®) and cDNA was prepared using RevertAid first strand cDNA synthesis kit (Fermentas) as per the manufactures instruction. Oligonucleotide primers (Table 1) were designed, synthesized and standardized for PCR reaction conditions. PCR amplification was carried out in 25 µl volume, containing 10 pM of each primer, 0.1mM dNTP mix, 1 unit of Taq DNA polymerase (Fermentas, U.S.A.) and 1 µl cDNA in 1x Taq polymerase buffer. Electrophoresis was performed on 2% agarose stained with ethidium bromide Semi-quantitative analysis was done using Image J analysis software. Expression of house keeping gene (28S rRNA) was used for normalization of data. The fold changes in the expression of genes were calculated using following formula.

\[
\text{Ratio target gene expression} = \frac{\text{Fold change in target gene expression (expt./control)}}{\text{Fold change in reference gene expression (expt./control)}}
\]

**STATISTICAL ANALYSIS**

Data collected from all the treatments were subjected to one way analysis of variance (ANOVA) using standard procedures described by Snedecor and Cochran (1980). Duncan

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

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Gene</th>
<th>Primer (5‘- 3‘)</th>
<th>Annealing Temp. (°C)</th>
<th>Size of amplicon (bp)</th>
<th>Putative biological role</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Insulin-like growth factor-I (IGF-I)</td>
<td>F-GGTGCTGAGCTGGTTGATGC R-CGTACAGAGCTGCAAGTTAGGT</td>
<td>58</td>
<td>203</td>
<td>Growth</td>
<td>JN942578</td>
</tr>
<tr>
<td>2</td>
<td>Insulin-like growth factor-II (IGF-II)</td>
<td>F-GGCCGCAGCACCACG A R-CCCGGCAGCAAAGT</td>
<td>58</td>
<td>215</td>
<td>Growth</td>
<td>JN942579</td>
</tr>
<tr>
<td>3</td>
<td>Interleukin-6 (IL-6)</td>
<td>F-GAAATCCCTCCCTGCAACATG R-TGAAACGGAACAACACTG</td>
<td>55</td>
<td>219</td>
<td>Humoral Immunity</td>
<td>JN639847</td>
</tr>
<tr>
<td>4</td>
<td>Tissue Necrosis Factor –alpha (TNF)</td>
<td>F-AGACCGATGGGAAGGAATGAA R-GAAGAGGCCCACACCACGACAG</td>
<td>55</td>
<td>219</td>
<td>Humoral Immunity</td>
<td>JN942589</td>
</tr>
<tr>
<td>5</td>
<td>Interleukin-2 (IL-2)</td>
<td>F-CCCGCTGCTAATCTGCTG R-TGAGACACCACGGAAACCATG</td>
<td>57</td>
<td>287</td>
<td>Cell mediate immunity</td>
<td>HE608819</td>
</tr>
<tr>
<td>6</td>
<td>Interleukin-12 (IL-12)</td>
<td>F-GCCGACTGAGATGTCCTG G A R-CCTTGGTTATTTTAACTG</td>
<td>57</td>
<td>227</td>
<td>Cell mediate immunity</td>
<td>JN942590</td>
</tr>
<tr>
<td>7</td>
<td>28S (Reference)</td>
<td>F-CAGGCTGAGCTGGTTGATG R-GCTCCCGCTGGT</td>
<td>58</td>
<td>273</td>
<td>House keeping</td>
<td>JN639848</td>
</tr>
</tbody>
</table>
Multiple Range Test (Duncan, 1955) was used for verifying the significance of the difference between treatment means.

RESULTS AND DISCUSSION

The chicks receiving in ovo injection of Zn did not show any difference in the expression of IGF-1 on 18th day of incubation and day of hatch, but the expression was reduced on 20th day of incubation. Expression of IGF-1 was significantly enhanced (P<0.01) on 3rd day post-hatch and thereafter the expression was reduced compared to un-injected control chicks. Higher expression of IGF-2 gene in in ovo Zn injected embryos on 18th (P<0.001) and 20th (P<0.001) day of incubation was observed, but the expression reduced on day of hatch and 3rd day post- hatch. Again the expression of IGF-2 was significantly higher on 7th day (P<0.05) post-hatch and thereafter it reduced significantly on 10th and 14th day post-hatch compared to un-injected control chicks. Higher expression of IGF-2 during late embryonic age could be due to the availability of in ovo supplemented zinc which might have been consumed from the yolk until E17 (Yair and Uni, 2011).

IGF-1 expression in in ovo I injected embryos/chicks was significantly higher during pre-hatch (18th day), day of hatch and 3rd day post-hatch compared to un.injected control chicks. Expression of IGF-2 on 20th day (P<0.001) of incubation was also higher in iodine injected embryos. AS reported earlier (Tsukada et al., 1998) thyroid hormones regulates IGF-I production in the chicken. Higher expression of IGF-1 during late incubation might be due to extra availability of I playing an important role in stimulating thyroid hormone which is further involved in the differentiation and final maturation of many tissues just prior to hatching (Black, 1978). However, no difference was observed in the expression of IGF-1 and IGF-2 gene on 7th, 10th and 14th day post-hatch.

While studying the expression of humoral

![Graph 1](image1.png)

**Figure 1** - Mean fold increase of growth related at 18th and 20th day embryonic stage, Date of hatch 3rd, 7th, 10th and 14th day post hatch.

![Graph 2](image2.png)

**Figure 2** - Mean fold increase of Immunity related genes.
immune related genes there was an up regulation of IL-6 gene (P<0.01) in iodine injected chicks which is in confirmation with the earlier studies of Haddad and Mashaly (1990) who reported that relative bursa weights were greater in chicks that received T3 or TRH and increased total WBC count. No difference was observed in TNF-α gene expression in both the treatments however Zinc treatment has shown down regulation of IL6 gene.

The cell-mediated immunity was possibly related to the production of interleukin-2, which was supported by higher Zn level in the diet (Kidd et.al 1996; Shyam Sunder et.al 2008). In our study the expression of IL-2 (P<0.002) and IL-12 (P<0.04) gene was significantly increased in zinc injected chicks. This is in line with the earlier study of Bartlett and Smith (2003) who reported that the immune response of broilers can be influenced by the level of zinc in the diet. Our results also correlate the finding of Haddad and Mashaly (1990) who suggested that TRH released from Thyroid does not have any effect on splean as in our study also iodine treatment had no effect on IL-2 and 12 gene expressions.

CONCLUSION

It is concluded that both zinc and iodine modulate growth related genes during late embryonic stage. Iodine plays a role in humoral immunity, while zinc has role in cellular immunity.

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