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Supplementation of inorganic and organic forms of zinc, selenium and chromium on immune responses in broiler chicken

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Abstract
Poultry production is one of the rapidly growing sub sectors among the various livestock sectors in India. Besides contributing markedly to GDP and earning foreign exchange, it also produces good quality animal protein at an affordable price to meet the protein demand of human population by exploiting the genetic potential and adopting the standard scientific managemental and nutritional practices. Hence, in the present investigation we explored the use organic zinc, selenium and chromium to enhance immune response in broiler chickens. Total 312 day old Cobb broiler chicks were used in the experiment. Broilers were divided into 13 groups and each group consisting of 24 birds in 3 replicates. T1 group was kept as control. T2, T3, T4 group was supplemented with zinc (40 mg/kg of feed) from inorganic, 50% inorganic + 50% organic and organic form respectively. T5, T6, T7 group was supplemented with selenium (0.3 mg/kg of feed) from inorganic, 50% inorganic + 50% organic and organic form respectively. T8, T9, T10 group was supplemented with chromium (2 mg/kg of feed) from inorganic, 50% inorganic + 50% organic and organic form respectively. T11, T12, T13 group was supplemented with combination of all 3 minerals from inorganic, 50% inorganic + 50% organic and organic form respectively.
The mean concentration of plasma IgG and corticosterone concentration showed non-significant difference between control and treatment groups in broiler. RT-PCR expression analysis of IL 10 gene revealed that maximum upregulation (9.21 fold) was found in T7 group, followed by T10 (4.80 fold) and T4 (4.6 fold) in spleen tissue as compared to control group. Mean heterophil and lymphocyte ratio was significantly ($p<0.05$) higher in T1 group as compared to control and other treatment group. In the present investigation organic Zn, Se and Cr supplementation improved immune and anti-inflammatory status, which could be translated into better production performance and lower mortality.

Keywords: inorganic and organic, zinc, selenium and chromium and broiler chicken

Introduction
Nearly one third of world-wide broiler and layer stock placement is in Asian countries such as India and China and these countries are emerging as important locations for the production and trade of poultry product (Mishra *et al*., 2015) [15]. Poultry farming in India has achieved a tremendous growth rate in last four and half decades, from an age old backyard farming to most sophisticated agro based industry. Advances in broiler chicken genetics, nutrition and management have resulted in improved performance of birds. Yet, a higher metabolic rate has made chickens prone to external factors that can alter their welfare, productivity, health or even cause death. Among the external factors causing immunodeficiency are high stocking density, extreme temperature, long lighting programs, diseases and toxins. Utilization of effective trace minerals in diet will protect the biological system by enhancing immune responses.
Zinc (Zn), selenium (Se) and chromium (Cr) act as catalysts in many enzyme and hormone systems (Rama Rao *et al*., 2016) [20]. Conventionally, inorganic minerals are used in chicken diet, because they are cost- effective and readily available, but are relatively inferior to organic minerals due to poor bioavailability (Virden *et al.,* 2004) [29]. Higher concentrations of inorganic TM (ITM) will interfere with each other which may cause either deficiency or toxicity. However, organic form of TM (OTM) will not interfere with other minerals due to different pathway of absorption through intestinal wall (Rama Rao *et al*., 2016) [20].
Supplementation of zinc (Zn) in broiler diet is of particular interest because they function predominantly as catalyst in many enzyme and hormone system that are associated with

growth, immune response and have antioxidant activity. The Zn was found to be essential for normal functioning of the immune system by increasing the counts of thymocytes and peripheral T cells and activity of natural killer cells. It also enhances the production of neutrophils and antibodies, in addition to improving the functions of macrophages (Sunder *et al*., 2008) [25]. Chromium supplementation in diet has been related to increased protein deposition, with decrease in muscle fat. Immunological function has been enhanced by trivalent Cr and its effects seem more pronounced during times of stress. Selenium influences immune responses through its incorporation into selenoproteins such as the amino acid selenocysteine (Hoffmann, 2007) [11]. Therefore, selenium (Se) plays an important role in immune function, production of immunoglobulin.

In mammals, IL-10 is a 17 to 19-kDa cytokine containing two disulfide bridges that functions as a homodimer. It is produced by activated T cells, B cells, monocytes/macrophages, mast cells and keratinocytes. The predominant effect of IL-10 in mammals is to modify immune responses through direct effects on many cell types, including T cells, B cells, APCs and NK cells (Ding *et al*., 2003) [4].

Hence, the present study has been designed to study the influence of inorganic and organic source of zinc, selenium and chromium supplementation on immune response of broilers.

Materials and Methods

The research was carried out in the Department of Veterinary Physiology and Biochemistry, College of Veterinary Science and Animal Husbandry, N.D.V.S.U., Jabalpur (M.P.).

Experimental birds

Three hundred and twelve (312) day old Cobb broiler chicks were procured from Private hatcheries of Jabalpur. The birds were maintained in the battery cage system in a well ventilated room in the poultry experimental unit at college with prior permission from Institutional Animal Ethics Committee. Broilers were divided into 13 groups and each group consisting of 24 birds in 3 replicates. T1 group was kept as control. T2, T3, T4 groups were supplemented with zinc (40 mg/kg of feed) from inorganic, 50% inorganic + 50% organic and organic form respectively. T5, T6, T7 groups were supplemented with selenium (0.3 mg/kg of feed) from inorganic, 50% inorganic + 50% organic and organic form respectively. T8, T9, T10 groups were supplemented with chromium (2 mg/kg of feed) from inorganic, 50% inorganic + 50% organic and organic form respectively. T11, T12, T13 groups were supplemented with combination of all 3 minerals from inorganic, 50% inorganic + 50% organic and organic form respectively.

Diets were formulated as per NRC (1994) [16] specifications. Feed-grade sulphate salts of Mn, Zn, Fe and Cu were used in the control diet (CD), while in inorganic treatment group Se and Cr were used in the form of selenite (sodium selenite) and dichromate (potassium dichromate), respectively. The organic forms of Se, Zn and Cr (Sel-Plex 2000, Bioplex zinc and Biochrome, respectively) were generous gift from Alltech Biotechnology Pvt. Ltd., Bengaluru, India. Concentrations of Zn, Se and Cr in the above organic TM premixes were 15, 0.2 and 0.1%, respectively.

Experimental design

Groups	Replicates	No. of Broilers	Treatments
T_1 (n=24)	T_1R_1 T_1R_2 T_1R_3	8 8 8	Basal diet
T_2 (n=24)	T_2R_1 T_2R_2 T_2R_3	8 8 8	Basal diet + Inorganic zinc @ 40 mg/kg of feed
T_3 (n=24)	T_3R_1 T_3R_2 T_3R_3	8 8 8	Basal diet + 50% Inorganic zinc @ 20 mg/kg of feed+ 50% Organic zinc @ 20 mg/kg of feed
T_4 (n=24)	T_4R_1 T_4R_2 T_4R_3	8 8 8	Basal diet + Organic zinc @ 40 mg/kg of feed
T_5 (n=24)	T_5R_1 T_5R_2 T_5R_3	8 8 8	Basal diet + Inorganic selenium @ 0.30 mg/kg of feed
T_6 (n=24)	T_6R_1 T_6R_2 T_6R_3	8 8 8	Basal diet + 50% Inorganic selenium @ 0.15 mg/kg of feed + 50% Organic selenium @ 0.15 mg/kg of feed
T_7 (n=24)	T_7R_1 T_7R_2 T_7R_3	8 8 8	Basal diet + Organic selenium @ 0.30 mg/kg of feed
T_8 (n=24)	T_8R_1 T_8R_2 T_8R_3	8 8 8	Basal diet + Inorganic chromium @ 2 mg/kg of feed
T_9 (n=24)	T_9R_1 T_9R_2 T_9R_3	8 8 8	Basal diet + 50% Inorganic chromium @ 1 mg/kg of feed + 50% Organic chromium @ 1 mg/kg of feed
T_{10} (n=24)	$T_{10}R_1$ $T_{10}R_2$ $T_{10}R_3$	8 8 8	Basal diet + Organic chromium @ 2 mg/kg of feed
T_{11} (n=24)	$T_{11}R_1$ $T_{11}R_2$ $T_{11}R_3$	8 8 8	Basal diet + Inorganic zinc @ 40 mg/kg of feed + Inorganic selenium @ 0.30 mg/kg of feed + Inorganic chromium @ 2 mg/kg of feed
T_{12} (n=24)	$T_{12}R_1$ $T_{12}R_2$	8 8	Basal diet + 50% Inorganic zinc @ 20 mg/kg of feed+ 50% Organic zinc @ 20 mg/kg of feed + 50% Inorganic selenium @ 0.15 mg/kg of feed + 50% selenium @ 0.15 mg/kg of feed +50%

	T_{12} R_3	8	Inorganic chromium @ 0.15 mg/kg of feed + 50% chromium @ 0.15 mg/kg of feed
T_{13} (n=24)	T_{13} R_1 T_{13} R_2 T_{13} R_3	8 8 8	Basal diet + organic zinc @ 40 mg/kg of feed + organic selenium @ 0.30 mg/kg of feed + organic chromium @ 2 mg/kg of feed

Table 1: Ingredients and composition of broiler ration

Ingredients	Starter %	Finisher %
Maize	43.36	57.30
Soybean meal	43.90	33.10
Soybean oil	8.74	5.61
Common Salt	0.40	0.40
DL- Methionine	0.185	0.175
Di-Calcium Phosphate	1.80	1.80
Limestone Powder	1.37	1.37
Supplements (Vitamins supplement and feed additives)	0.245	0.245

- *Trace mineral Premix: Mn-55,I-0.4, Fe-56 and Cu-4kg-1
- ** Vitamin premix: Vitamin A-8250 IU, Vitamin D_3- 1200 IU, Vitamin k-1mg, Vitamin E-40 IU, Vitamin B1-2mg, Vitamin B_2-4mg, Vitamin B_{12}-10mg, Percent of values specified by NRC, 1994 [16], *** Calculated

Sample Size

The physiological, humoral immune response, hormonal, heterophil – lymphocyte ratio were recorded on all the experimental birds. The molecular analysis was done for T1, T4, T7, T10 and T13 group which were supplemented with organic form of Zn, Se and Cr. Birds were sacrificed on day 35 of the experiment and organs including the ileum, caecal tonsils, bursa of fabricius and spleen were collected for IL 10 gene expression analysis.

Collection of samples

Blood samples (approx. 2 ml) were collected on day 21, 28 and 35 from each bird of all experimental groups. Blood were collected from the wing vein. The blood samples collected in heparinized polypropylene tubes (20 IU heparin/ml of blood) were kept in the ice bucket and carried back to the laboratory immediately. In the laboratory, all the blood samples were centrifuged at 3000 rpm for 30 min and plasma was separated. Plasma obtained was kept in the labeled storage vials of 2 ml capacity and stored at -20 °C till analysis of plasma IgG, chicken corticosterone.

The quantitative estimation of chicken IgG levels and plasma corticosterone were analyzed by the sandwich Elisa technique.

IL 10 gene expression analysis studies

Total RNA was isolated from the liver following standard TRIzol method. The purity of RNA was checked before the preparation of first- strand cDNA. Prepared cDNA was stored at -20 °C and later used for IL 10 gene expression studies. Expression of IL 10 gene was quantifed using gene specifc primer pairs using Real–Time PCR. β-actin was used as a reference gene.

RNA extraction

The birds were sacrifced following the appropriate standard procedure. Aseptically, tissue samples were collected from broiler birds for isolation of RNA. Total RNA was isolated from tissues samples of birds using TRIzol reagent (Sigma-aldrich, USA).

First strand cDNA synthesis

The frst strand cDNA was synthesized using Revert Aid TM frst strand cDNA synthesis kit (MBI Fermentas).

Primers

Primers for IL 10 gene and β-actin (β-actin; used as housekeeping gene) were adopted from Echeverry *et al*., 2016 [5]. Sequence of gene specific primers for IL 10 gene and β-actin are as follows:

S. No	Gene	Primers	Annealing Temperature	Gene bank access
1	chIL-10	F: AGCAGATCAAGGAGACGTTC R: ATCAGCAGGTACTCCTCGAT	55 ºC	AJ621614
2	Chβ-actin	F:CAACACAGTGCTGTCTGGTGGTA R: ATCGTACTCCTGCTTGCTGATCC	61 ºC	X00182

PCR reaction mixture

Readymix Taq PCR Reaction mix with MgCl2 (Sigma Aldrich, U.S.A.) was used to prepare PCR reaction mixture of 20 µl (2 X Ready Mix Taq PCR Reagent Mix - 10 µl, forward primer (10 pM)- 0.1 µl, reverse primer (10 pM)- 0.1 µl and cDNA 1 µl) total volume was prepared and run in the thermal cycler (Bio-Rad laboratories Inc. USA). The PCR protocol designed for 35 cycles is as follows and it was kept same for both the primers used:

S. No.	Steps		chIL-10	Chβ-actin
1.	Initial Denaturation	Temperature	94 °C	94 °C
		Time	10 min	10 min
2.	Denaturation	Temperature	94 °C	94 °C
		Time	1 min	1 min
3.	Annealing	Temperature	58 °C	58 °C
		Time	45 sec	45 sec
4.	Extension	Temperature	72 °C	72 °C
		Time	1 min	1 min
5.	Final Extension	Temperature	72 °C	72 °C
		Time	10 min	10 min
6.	Hold	Temperature	4 °C	4 °C
		Time	∞	∞

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR/Real Time PCR)

The relative expression of gene specifc mRNA was quantifed by qRT-PCR/Real-time PCR employing SYBR green chemistry (CFX Connect Real-time System, Bio Rad laboratories Inc. USA).

Relative quantification Comparative CT method (Livak and Schmittgen, 2001) [14] was used for relative expression of target gene in the test sample relative to that of control sample (calibrator- T1). The relative expression of target genes was estimated in term of fold change in mRNA expression, using the following formula:

Fold change in expression of target gene = $2^{-\Delta\Delta C}$T where,

ΔΔ CT = Δ CT test - ΔCT control/calibrator

Δ CT test = CT target gene - CT reference gene (In test / treatment group)

ΔCT control/calibrator = CT target gene – CT referenc gene (In control/calibrator group)

where, CT target gene = mean of the cycle threshold (CT) values of the gene being tested

CT reference gene = mean of the CT value of the housekeeping gene β-actin

Immune organ weight of broilers

After 35 days of age, two birds with the average body weight per group were sacrificed by cervical dislocation. Immediately after bleeding from the jugular vein, several organs including the ileum, caecal tonsils, bursa of fabricius and spleen were harvested and weighed individually

Heterophill / lymphocyte ratio (H:L)

Heterophil and Lymphocyte was counted by staining the blood smears by Leishman's stain and manually counting as per the method outlined by Feldman and Jain (2002) [6]. At least 100 cells were counted.

Statistical analysis

The recorded data was statistically analyzed using Completely Randomized Design (Snedecor and Cochran, 1994) [24]. Various conditions and treatment groups were compared by using Duncan Multiple Range test (DMRT).

Results and Discussion

Chicken plasma IgG concentration

Chicken plasma IgG concentration in broilers has been presented in Table 02. The mean concentration of plasma IgG showed non-significant difference between all the groups. However, on day 21 the maximum increment 99.57% of plasma IgG was found in T13 (7.781 ± 0.70 µg/ ml) group followed by T12 (80.87%) than T4 (78.87%) group whereas, the minimum concentration of plasma IgG was found in T1 (3.903 ± 1.09 µg/ ml) group. On day 28 the maximum increment 65.42% of plasma IgG was found in T13 (10.493 ± 2.23 µg/ ml) group followed by T12 (45.71%) than T4 (38.51%) group and the minimum concentration of plasma IgG was found in T1 (6.343 ± 0.47 µg/ ml). On day 35, the maximum increment 60.65% of plasma IgG was found in T13 (10.507 ± 1.18 µg/ ml) group followed by T12 (42.35%) group and the minimum concentration of plasma IgG was found in T1 (6.540 ± 1.14 µg/ ml) i.e. control group.

Table 2: Mean Plasma IgG concentration (µg/ ml) in broilers at different intervals

Period / Treatment	21st day	28th day	35th day
T1	3.903 ±1.09	6.343 ± 0.47	6.540 ± 1.14
T2	5.293 ± 0.78	7.026 ± 0.89	8.403 ± 1.03
T3	6.403 ± 0.60	8.484 ± 0.62	8.408 ± 1.19
T4	6.976 ± 1.53	8.786 ± 0.44	8.595 ± 1.80
T5	4.454 ± 1.47	6.873 ± 1.56	7.131 ± 1.07
T6	6.012 ± 1.63	8.022 ± 0.80	8.349 ± 1.08
T7	6.943 ± 0.30	8.185 ± 0.67	8.472 ± 1.86
T8	4.278 ± 2.12	6.644 ± 1.63	7.243 ± 0.74
T9	5.335 ± 0.20	7.979 ± 1.00	7.653 ± 0.97
T10	6.362 ± 1.40	8.120 ± 0.25	8.570 ± 1.27
T11	6.623 ± 0.55	8.604 ± 0.25	8.618 ± 1.45
T12	7.054 ± 0.48	9.243 ± 0.41	9.310 ± 1.58
T13	7.781 ± 0.70	10.493 ± 2.23	10.507 ± 1.18

The mean concentration of plasma IgG showed non-significant difference between all the groups. However, the maximum concentration of plasma IgG was found in T13 group whereas, the minimum concentration of plasma IgG was found in T1 group during the entire investigation period. Similar, results were reported by Rama Rao *et al.* (2016) [20], where supplementation of organic Zn, Se and Cr did not significantly affect the antibody titre of ND vaccine. Contrary to these findings, Ghazi *et al.* (2012) [8] reported that organic mineral forms resulted in significantly higher titers of both IgM and IgG as compared to inorganic mineral forms in heat stressed broiler chickens. Uyanik *et al.* (2002) [28] showed that IgG and IgM antibody titers in both primary and secondary immune responses to SRBC were improved by increasing level of CrCl3 (2,000, 4,000 or 8,000 µg kg-1). Toghyani *et al.* (2007) [27] reported that dietary supplementation of Cr 1500 ppb tended to of 1500 ppb significant ($p<0.05$) increase in IgG concentration at day 42.

The lack of immune responses in the present study might be due to variation in the dose of the minerals used. Also, in many reports it has been observed that stress causes suppression of antibody production in young chickens. This reduction could be indirectly due to an increase in inflammatory cytokines under stress, which stimulates the hypothalamic production of corticotrophin releasing factor (Ogle *et al.*, 1997) [17]. Corticotrophin releasing factor is known to increase adrenocorticotropic hormone from the pituitary; which inturn stimulates corticosterone production from the adrenal gland. It has been well documented that corticosterone inhibits antibody production. The higher response observed might be due to use of relatively higher concentrations of Zn (up to 181 mg/kg) as compared to the levels used in the present study (20 and 40 mg Zn/kg). The data obtained in the present investigation showed same pattern as obtained by Ogle *et al.* (1997) [17]. It has been reported that under heat stress conditions, supplementation of chromium (organic or inorganic) significantly improved antibody titres against ND vaccine. Sajadifar *et al.* (2013) [22] also reported higher requirement of Zn (120 and 200 mg/kg) for augmentation of humoral immune response in chickens.

Present findings were in accordance with other workers (Hegazy and Adachi, 2000 and Denghua *et al.*, 2001) [10, 3]

who reported an increase in humoral antibody titers, when selenium was used in feed. The perceptible reason for enhanced antibody production might be the increase in number of lymphocytes with increased selenium supplementation.

4.3 Hormonal Analysis

4.3.1 Chicken corticosterone estimation

Chicken plasma corticosterone concentration in broilers has been presented in Table 03. The mean concentration of plasma corticosterone showed non-significant difference between all the groups. On day 21, the maximum concentration of plasma corticosterone was found in T1 (7.680 ± 2.14 ng/ ml) group and the maximum reduction 61.41% of plasma corticosterone was found in T13 (2.963 ± 1.31 ng/ ml) group. On day 28, the maximum concentration of plasma corticosterone was found in T1 (7.406 ± 0.51 ng/ ml) group and the maximum reduction 47.93% was found in T13 (3.856 ± 0.38 ng/ ml). On day 35, the maximum concentration of plasma corticosterone was found in T1 (7.510 ± 1.12 ng/ ml) group and the maximum reduction 52.69% was found in T13 (3.553 ± 0.92 µg/ ml) group. The results showed non significant difference in plasma corticosterone concentration among the different treatment groups.

Table 3: Mean plasma corticosterone concentration (ng/ ml) in broilers at different intervals

Period / Treatment	21st day	28th day	35th day
T1	7.680 ± 2.14	7.406 ± 0.51	7.510 ± 1.12
T2	5.537 ± 1.25	5.736 ± 1.28	5.811 ± 1.74
T3	4.773 ± 0.12	4.740 ± 1.67	4.876 ± 0.60
T4	4.553 ± 0.20	4.573 ± 0.55	4.581 ± 0.53
T5	5.583 ± 1.00	5.993 ± 1.87	5.675 ± 0.85
T6	4.953 ± 0.29	5.030 ± 0.72	5.333 ± 0.54
T7	4.556 ± 2.10	4.640 ± 0.11	4.795 ± 0.55
T8	6.936 ± 2.13	6.326 ± 1.83	6.362 ± 1.63
T9	6.106 ± 1.06	5.623 ± 1.08	3.417 ± 1.15
T10	4.760 ± 0.46	4.696 ± 0.28	4.689 ± 1.17
T11	4.160 ± 0.45	4.500 ± 0.18	4.646 ± 0.50
T12	3.473 ± 0.69	4.306 ± 0.24	4.337 ± 1.09
T13	2.963 ± 1.31	3.856 ± 0.38	3.553 ± 0.92

Chicken plasma corticosterone showed non significant difference among the different treatment groups, but their levels were slightly reduced by the supplementation of organic trace minerals. The minimum concentration of plasma corticosterone was found in T13 group whereas, the maximum concentration of plasma IgG was found in T1 group during the entire experimental period. The probable reason for this might be the release of corticosterone as an adaptogenic response with organic Zn, Se and Cr.

Kegley *et al.* (1964) [12] reported that serum cortisol concentrations were non significantly affected by chromium supplementation on day 27. Arthington *et al.* (1997) [1] reported that treatment with Cr does not affect the secretion of ACTH, cortisol or plasma tumor necrosis factor-a, although clear circadian variation in ACTH and cortisol occurred. Similar results were also observed in the present research investigation.

Bahrami *et al.* (2012) [2] reported that serum cortisol concentrations lowered significantly ($p<0.01$) on day 28 and 42 in the 1,200 ppb Cr-L-Met fed group, which is in disagreement to present reports but the pattern of corticosterone was same for both the studies.Therefore, supplementing the diet with organic minerals might help in the amelioration of stress in chickens.

4.4 Molecular analysis

4.4.1 Expression of IL 10 gene of broilers

The mRNA expression levels of IL10 gene on day 35, in spleen, caecal tonsil, bursa of fabricius and ileum sample of broiler birds has been presented in terms of fold change in expression in Table 04. In all the samples maximum up regulation of IL 10 gene was found in spleen. In the spleen samples, maximum up regulation was found in T7 (9.21 fold) followed by T10 and minimum up regulation was found in T13 (2.16 fold) group. In caecal tonsil, maximum up-regulation was found in T7 (3.90 fold) and minimum up regulation was found inT13 (1.13 fold) group. In bursa of fabricius maximum up regulation was found in T7 (3.58 fold) and minimum up regulation was found in T13 (1.39 fold) group. Same results were also found for ileum, where maximum up regulation was found in T7 (1.23 fold) and minimum up regulation was found in T13 (1.06 fold) group.

Table 4: Comparative gene expression profiling (fold change) of IL 10 gene in different treatment groups in spleen, caecal tonsil, bursa of fabricius and ileum in broilers

Organ / Treatment	Spleen	Caecal Tonsil	Bursa of fabricius	Ileum
T1	1	1	1	1
T4	4.69	2.51	1.81	1.12
T7	9.21	3.90	3.58	1.23
T10	4.80	1.75	1.83	1.09
T13	2.16	1.13	1.39	1.06

The mRNA expression level of IL 10 gene was up-regulated in all the organic mineral supplemented groups as compared to control group which indicated the ameliorative effects of organic mineral supplementation in broilers. The maximum up-regulation was found in spleen of selenium supplemented group. The probable reason for this may be due to more number of activated cells in circulation of organic selenium supplemented group of broilers. IL 10 is a pleiotropic cytokine, known to be an important regulator of lymphoid and myeloid effector functions. Interleukin 10 downmodulates phagocytic immune responses and accentuates humoral responses. The IL-10 gene contains several putative transcriptional control motifs including glucocorticoid responsive elements, a cAMP responsive element (Le *et al*., 1997) [13] which may be the possible reason behind up-regulation of IL 10 gene expression in present investigation. Glucocorticoid is directly related to the level of stress in body. Therefore, the up-regulation of anti-inflammatory IL-10 expression in the spleen to a lesser extent can help to infer an anti-inflammatory effect of OTM.

Echeverry *et al.* (2016) [5] reported that organic trace mineral supplementation significantly ($p<0.05$) up-regulate IL 10 gene in spleen as compared to control group, which is in agreement to present findings.

4.5 Immune organ weight of broilers

The weight of spleen, caecal tonsil, bursa of fabricius and ileum has been presented in Table 05. Maximum weight of spleen was found in T13 (2.18 g) followed by T7 (2.10 g) group and minimum was found in T1 (1.45 g) group. Same pattern of results were found for caecal tonsil, bursa of fabricius and ileum. Maximum weight of caecal tonsil was found in T13 (0.41 g) and minimum was found in T1 (0.28 g)

group. In bursa of fabricius maximum weight was found in T13 (1.89 g) group and minimum was found in T1 (1.45 g) group. In ileum Maximum weight was found in T13 (1.05 g) group and minimum was found in T1 (0.73 g) group.

Table 5: Immune organ weight (g) of broilers

Organ / Treatment	Bursa of Fabricius	Spleen	Ileum	Caecal Tonsil
T1	1.12	1.45	0.73	0.28
T2	1.20	1.52	0.77	0.31
T3	1.35	1.61	0.82	0.33
T4	1.54	1.73	0.87	0.35
T5	1.28	1.56	0.78	0.32
T6	1.44	1.84	0.96	0.36
T7	1.60	2.10	0.99	0.41
T8	1.18	1.50	0.74	0.30
T9	1.36	1.73	0.89	0.34
T10	1.52	1.98	0.97	0.38
T11	1.44	1.67	0.83	0.38
T12	1.51	1.91	0.98	0.40
T13	1.89	2.18	1.05	0.41

Maximum weight of bursa of fabricius, spleen, ileum and caecal tonsil were found in T13 group followed by T7 group and minimum in T1 group which is directly correlated to the body weight gain in the present research work.

Osman and Ragab (2007) [18] reported that broiler chicks fed diet supplemented with 0.3 g/kg Zn methionine had the highest spleen percentage. Sunder *et al*. (2008) [25] found that birds fed with supplemental Zn at 40 ppm had significantly higher weight of spleen as compared with birds fed lower levels. Feng *et al*. (2010) [7] found that indices for spleen and bursa of fabricius increased linearly with increasing dietary Zn-Gly levels. Gheisari *et al.* (2011) [9] reported that different levels and sources of zinc, copper and manganese increased the relative weight of spleen and bursa of fabricius which is in accordance to present finding. Bahrami *et al.* (2012) [2] reported that supplemental dietary Cr, particularly at the level of 1,200 ppb of Cr-l-Met, improved body weight gain and weight of the lymphoid organs, but not significantly, which is in agreement to present findings.

Contrary to these findings Toghyani *et al.* (2007) [27] reported that the weights of lymphoid organs were not affected in chicks, fed diets with different levels of supplemental Cr. Yang *et al*. (2011) [30] found that supplementation of basal broiler diets with graded levels of trace minerals (Fe and Zn) had non significant effect on relative weight of spleen.

4.6 Heterophill / lymphocyte ratio (H: L)

The mean heterophill / lymphocyte ratio of broilers has been presented in Table 06. The mean heterophill / lymphocyte ratio showed significant difference ($p<0.01$) between all the groups.

On day 21, significantly ($p<0.01$) higher heterophill / lymphocyte ratio was observed in T1 (0.47 ± 0.05), T5 (0.45 ± 0.02) and T8 (0.45 ± 0.03) group. These groups differ significantly ($p<0.01$) from all other group except T9 group. T2, T3, T4, T6, T7, T10, T11, T12 and T13 groups differ non significantly from each other.

On day 28, significantly ($p<0.01$) higher heterophill / lymphocyte ratio was observed in T1 (0.52 ± 0.03) group. T2, T5 and T3, T6 groups were non significantly different from each other. T4, T7, T10, T11, T12 and T13 group differ non significantly from each other but were significantly ($p<0.01$) different from other group.

On day 35, significantly ($p<0.01$) higher heterophill / lymphocyte ratio was observed in T1 (0.52 ± 0.02), T6 (0.47 ± 0.01), T18 (0.51 ± 0.02) and T9 (0.50 ± 0.01) groups. However, significantly ($p<0.01$) lower heterophill / lymphocyte ratio was found in T13 (0.23 ± 0.01) group. Further, T12 and T13 group were non significantly different from each other, whereas T2, T3, T5 and T11 group differ non significantly from each other.

Table 6: Mean heterophil : lymphocyte ratio in broilers at different intervals

Period / Treatment	21st day	28th day	35th day
T1	$0.47^{a} \pm 0.05$	$0.52^{a} \pm 0.03$	$0.52^{a} \pm 0.02$
T2	$0.33^{bc} \pm 0.01$	$0.41^{bc} \pm 0.02$	$0.43^{bc} \pm 0.02$
T3	$0.32^{bc} \pm 0.04$	$0.34^{de} \pm 0.02$	$0.39^{c} \pm 0.01$
T4	$0.31^{bc} \pm 0.03$	$0.29^{ef} \pm 0.01$	$0.30^{d} \pm 0.03$
T5	$0.45^{a} \pm 0.02$	$0.42^{bc} \pm 0.02$	$0.43^{bc} \pm 0.02$
T6	$0.35^{bc} \pm 0.01$	$0.33^{de} \pm 0.02$	$0.47^{ab} \pm 0.01$
T7	$0.31^{bc} \pm 0.02$	$0.30^{ef} \pm 0.03$	$0.30^{d} \pm 0.02$
T8	$0.45^{a} \pm 0.03$	$0.48^{b} \pm 0.04$	$0.51^{a} \pm 0.02$
T9	$0.39^{ab} \pm 0.02$	$0.37^{cd} \pm 0.02$	$0.50^{a} \pm 0.01$
T10	$0.32^{bc} \pm 0.02$	$0.30^{def} \pm 0.02$	$0.32^{d} \pm 0.02$
T11	$0.31^{bc} \pm 0.03$	$0.31^{def} \pm 0.01$	$0.39^{c} \pm 0.01$
T12	$0.29^{c} \pm 0.03$	$0.27^{ef} \pm 0.04$	$0.27^{de} \pm 0.01$
T13	$0.28^{c} \pm 0.03$	$0.25^{f} \pm 0.01$	$0.23^{e} \pm 0.01$

Means bearing different superscripts within same column differ significantly ($p<0.01$).

The mean heterophil: lymphocyte ratio showed significant difference ($p<0.01$) between all the groups. The higher heterophil: lymphocyte ratio was observed in control group which declined with the supplementation of organic trace minerals.

In the current study, increase in lymphocytes and decrease in heterophils count and consequently decrease in heterophil to lymphocyte ratios in broiler chicks fed with different level and form of organic Zn, Se and chromium were observed. Similar findings were also reported by Uyanik *et al*. (2002) [28] observed that chromium supplementation of the basal diet (0, 2,000, 4,000 or 8,000 µg kg-1 CrCl3) increased lymphocyte counts and decreased heterophil to lymphocyte ratio in SRBC-inoculated chicks. Toghyani *et al*. (2008) [26] also reported that heterophil to lymphocyte ratio were significantly decreased in heat stressed broiler chicks fed 1,000 and 1,500 µg kg-1 CrPic. Heterophils are particularly sensitive to stressors such as adrenocorticotropic hormone (ACTH). The probable reason for increase in lymphocytes and decrease in heterophils count and decrease in heterophil to lymphocyte ratio by organic Zn, Se and Cr supplementation in broilers in this study may be due to reduction in glucocorticoid secretions or higher production of IL-2.

Present finding also correlate with the findings of Sirirat *et al.* (2012) [23], they reported significant decrease in Heterophil: Lymphocyte ratio in broilers fed with nanoparticles of chromium picolinate. Rouhalamini *et al.* (2014) [21] observed significant decrease in Heterophil: Lymphocyte ratio by feeding zinc sulphate and chromium picolinate to Japanese quails. Contrary to these findings Echeverry *et al.* (2016) [5] reported that the H/L ratio was higher in the OTM treatment group as compared to control ($p<0.05$) which is in disagreement to present findings.

Conclusion

Supplementation of organic form of Zn, Se and Cr (40, 0.30 and 2 mg/kg, respectively) both alone or in combination increases humoral immune response and in turn decreases the

level of stress in broilers. Also, up-regulation of cytokine IL-10 gene expression in the spleen indicates beneficial effect of organic trace minerals in augmentation of immunological apparatus in broilers.

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