Comparative effects of inorganic and organic trace minerals (Zn, Se and Cr) supplementation on expression of ChTLR2b gene in broilers

Anand Kumar Jain, Aditya Mishra, AP Singh, RPS Baghel, RK Sharma and SN Shukla

DOI: https://doi.org/10.22271/chemi.2020.v8.i4ai.10083

Abstract
Poultry is the fastest growing livestock sector in the developing countries. The global poultry sector is expected to continue to grow, as demand for poultry meat is driven by growing needs, population, rising incomes and urbanization. The present experiment was conducted to investigate the effect of inorganic and organic trace minerals supplementation on expression of ChTLR2b gene in broilers. A total of 216 broilers randomly divided into twelve groups and each group consisting of 18 broilers in 3 replicates. T1 group was kept as control. T2, T3 and T4 group was supplemented with zinc (40 mg/kg of feed) inorganic, organic and 50% organic form respectively. T5, T6 and T7 groups was supplemented with selenium (0.3 mg/kg of feed) from inorganic, organic and 50% organic form respectively. T8, T9 and T10 groups was supplemented with chromium (2 mg/kg of feed) from inorganic, organic and 50% organic form respectively. T11 and T12 group was supplemented with combination of all 3 minerals from inorganic and organic form respectively. RT-PCR expression analysis of ChTLR2b gene in spleen revealed that maximum up regulation (3.8413 fold) was found in T3 group (supplemented with organic Zn @ 40 mg/kg of feed), followed by T4 (3.5325 fold) whereas in bursa of fabricius the maximum up regulation (2.8921 fold) was found in T6 group followed by T12 (2.5310 fold ) as compared to control group. Up-regulation of gene expression ChTLR2b in bursa of fabricius and spleen indicates beneficial effect of organic trace minerals in potentiation of immune system in broilers.

Keywords: broilers, ChTLR2b gene, spleen, bursa of fabricius and organic trace minerals

Introduction
Broiler production in tropical countries is generally suboptimal as indicated by the poor growth performance and high mortality rate (Jaiswal et al., 2017) [1]. Conventionally, inorganic trace minerals zinc (Zn), chromium (Cr) and selenium (Se) are used in chicken diet, because they are cost- effective and readily available, but are relatively inferior to organic trace minerals due to poor bioavailability (Virden et al., 2004) [10]. It is well established that organic trace minerals (OTMs) are environment- friendly because of their lower excretion rate and it remains long time in the gut consequently improves the growth performance (Leeson and Caston, 2008) [6]. Organic trace minerals (OTMs) have been used in the broiler industry in order to enhance the immune system to protect birds from the harmful effects of pathogenic microorganisms. Dietary immunomodulation has been introduced to the broiler industry as a strategy to control the pathogens and maintain the health of broilers (Yitbarek et al., 2012) [11]. Toll- like receptors (TLRs) which are types of pattern recognition receptors (PRR) are trans- membrane proteins expressed by cells of innate immunity as well as epithelial cells. Modulation of PRR expressed by cells of the innate immune system including macrophages and dendritic cells would be followed by production of cytokines IL-10 (Pragati et al., 2019) [9] some of which are involved in B cell development and antibody production (Hirayama et al., 2018) [2]. TLRs perform a vital role as sentinels of the innate immune system. TLRs also facilitate the development of adaptive immune responses. TLR2b are expressed in spleen and bursa (Kawasaki and Kawai, 2014) [5]. Therefore, in present study, use of TLR2b gene (immune regulatory genes) as a molecular marker to identify the immune-modulatory property of organic Zn, Cr and Se in poultry diets were studied.
Material and methods
The proposed 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.). A total of 216 broilers randomly divided into twelve groups and each group consisting of 18 broilers in 3 replicates. T1 group was kept as control. T2, T3 and T4 group was supplemented with zinc (40 mg/kg of feed) from inorganic, organic and 50% organic form respectively. T5, T6 and T7 groups was supplemented with selenium (0.3 mg/kg of feed) from inorganic, organic and 50% organic form respectively. T8, T9 and T10 groups was supplemented with chromium (2 mg/kg of feed) from inorganic, organic and 50% organic form respectively. T11 and T12 group was supplemented with selenium (0.3 mg/kg of feed) from inorganic, organic and 50% organic form respectively.

Diets were formulated as per NRC (1994) [8] specifications. Inorganic and organic Zn, Se and Cr were supplemented along with feed as per the treatment groups. Broilers were kept in closed ventilated system for 35 days during the experimental period. Expression profile analysis of TLR2b in spleen and bursa of fabricius was done on day 35 of the experiment using RT-PCR technique. The recorded data was statistically analyzed using Completely Randomized Design. Various conditions and treatment groups were compared by using Duncan Multiple Range test (DMRT).

RNA extraction
Isolation of RNA was done from aseptically collected tissue from broilers and these broilers were sacrificed following the appropriate standard procedure. TRizol reagent (Sigma–Aldrich, USA) was used to isolate total RNA from spleen and bursa of fabricius of broilers.

RNA quantification and DNase-1 treatment
The purity and concentration of the total RNA was assessed using Nanodrop Spectrophotometer (ND 1000, Thermo Scientific). The purity of the total RNA was confirmed by considering the ratios of OD values at 260 and 280nm between1.9-2.0. The integrity of RNA was checked on 1.0% agarose gel using 1x TBE as electrophoresis buffer. The RNA samples showing contamination with DNA was incubated with RNase-free DNase-1 (MBI Fermentas) at 37 °C for 30 min. (@1 U for 1µg Total RNA). The DNase was subsequently inactivated by incubation at 65 °C for 10 min after adding the 25mM EDTA (@1 µl for 1µg Total RNA. Purity and concentration of DNase-treated total RNA sample was determined using nanodrop spectrophotometer.

First strand cDNA synthesis
The first strand cDNA was synthesized using Revert AidTM first strand cDNA synthesis kit (MBI Fermentas). 1. The components of kit were thawed at room temperature, mixed and briefly centrifuged and then stored on ice immediately.
2. A 20 µl reaction volume was used for 5 µg of total RNA. The following reagents were added into a nuclease free microcentrifuge tube on ice in the indicated order:

| Reagent                              | Quantity  |
|--------------------------------------|-----------|
| Total RNA                            | 5 µg      |
| Random Hexamer primer                | 1 µl      |
| Nuclease free water                  | To 12 µl  |
| 5X Reaction buffer                   | 4 µl      |
| RibolockRNase inhibitor (20 U/ µl)   | 1 µl      |
| 10 mMdNTP mix                        | 2 µl      |
| RevertAid M-MuL V RT (200 U/ µl)     | 1 µl      |
| Total Volume                         | 20 µl     |

The obtained mixture was mixed gently and centrifuged briefly. Mixture incubated at 65 °C for 5 minutes and quick chilled on ice. The following components were added in indicated order:

3. The contents of the tube were mixed gently and centrifuged briefly and incubated for 5 minutes at 25 °C followed by 60 minutes at 42 °C.
4. The reaction was terminated by heating at 70 °C for 5 minutes.
5. The resultant cDNA was stored frozen at -20 °C till used.

Polymerase chain reaction (PCR) primers
Primers for TLR2b gene and β-actin (β-actin; used as housekeeping gene) were adopted from Echeverry et al. (2016) [1]. Sequence of gene specific primers for ChTLR2b gene and β-actin are as follows:

Concentration optimization of cDNA and primers
To optimize the concentration of cDNA and primer, PCR was carried out with two fold serial dilution of cDNA (x, x/2, x/4, x/8 and x/16) and different primer concentrations i.e. 10, 5 and 2.5 pM.
The relative expression of gene specific mRNA was reaction (qRT-PCR/Real Time PCR) Quantitative reverse transcriptase-polymerase chain future analysis. 

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR/Real Time PCR) The relative expression of gene specific mRNA was quantified by qRT-PCR/Real Time PCR employing SYBR green chemistry using Real-time PCR system (CFX Connect Real-time System, Bio-Rad laboratories Inc. USA). All reactions were performed in nuclease-free 8 tube-strips with optically clear flat caps (Axygen Scientific, Inc. USA). For each sample a dissociation curve (melting curve) was generated after completion of amplification to ascertain the specificity of amplification. A negative control containing all the ingredients except cDNA template (Non-template control; NTC) was set up invariably for each master mix made for conducting the reactions. The results were expressed as CT values of target and reference genes in test (treatment) and control (calibrator) samples.

Real time PCR reaction protocol 

PCR cycling conditions were: initial denaturation of 94 °C for 3 minutes, followed by 40 cycles of denaturation 94 °C for 30 seconds; annealing 58 °C for 30 seconds and extension 72 °C for 30 seconds.

Relative quantification 

Comparative CT method (Livak and Schmittgen, 2001) [7] was used for relative expression of target gene in the test sample (treatment) relative to that of control sample (calibrator). The mRNA expression of target gene in test sample was expressed as “n-fold up/down regulation” in relation to control sample. For estimation of relative expression of target gene by the comparative CT method, CT values of target gene in test and control sample were adjusted to the CT values of a reference gene (endogenous/internal control). In the present study ChTLR2b was the target gene whereas β-actin was taken as reference gene. The CT for the target gene (ChTLR2b) and the CT for the reference gene (β-actin) was determined for each test sample and the control sample. 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 = \(2^{\Delta\Delta CT}\) where, \(\Delta\Delta CT = \Delta CT\) test - \(\Delta CT\) control/calibrator 

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

\(\Delta CT\) control/calibrator = \(CT\) target gene - \(CT\) reference gene (In control/calibrator group) 

Where, 

CT target gene = mean of the cycle threshold (CT) value of the gene being tested CT reference gene = mean of the CT value of the housekeeping gene β-actin 

Results and discussion 

The mRNA expression levels of ChTLR2b gene on day 35, in spleen and bursa of fabricius sample of broiler birds has been presented in terms of fold change in expression in Table 06 and Figure 01. In all the samples maximum up regulation of ChTLR2b gene was found in bursa of fabricius. In the spleen samples, maximum up regulation was found in T5 (8.06 fold) followed by T9 and minimum up regulation was found in T2
(1.53 fold) group. In bursa of fabricius maximum up regulation was found in T9 (13.57 fold) and minimum up regulation was found in T8 (1.39 fold) group. Echeverry et al. (2016) [1] reported that organic trace mineral supplementation enhances local and systemic innate immune responses and modulates oxidative stress in broiler chickens. The gene expression analysis showed that OTM treatment resulted in no change in ChTLR2b expression among treatments, which is contrary to present findings. In the present investigation fold change expression of ChTLR2b gene was up regulated more in bursa of fabricius as compared to spleen, which might be explained by the fact that an additional proinflammatory response was induced by organic Se and blend of organic Zn, Cr and Se treatment groups. Expression of ChTLR2b, found in chickens normally occurs at an early stage of inflammation and acts as a chemo-attractant for chicken heterophils (Kaiser and Staheli, 2014) [4].

Table 6: Comparative gene expression profiling (fold change) of ChTLR2b gene in different treatment groups in spleen and bursa of fabricius of broiler

| Gene Treatment | Bursa of fabricius | Spleen     |
|----------------|--------------------|------------|
| T1             | 1.0000             | 1.0000     |
| T2             | 1.6259             | 2.9956     |
| T3             | 2.2514             | 3.8413     |
| T4             | 1.8512             | 3.5325     |
| T5             | 1.9251             | 2.9856     |
| T6             | 2.8921             | 3.3351     |
| T7             | 1.8114             | 2.3622     |
| T8             | 1.8321             | 2.2515     |
| T9             | 1.9147             | 2.4210     |
| T10            | 1.7894             | 2.3324     |
| T11            | 1.9143             | 2.6141     |
| T12            | 2.5310             | 3.1127     |

**Fig 1:** Comparative gene expression profiling (fold change) of ChTLR2b gene in different treatment groups in spleen and bursa of fabricius of broiler.

**Conclusion**
In all the samples maximum up regulation of ChTLR2b gene was found in bursa of fabricius. In the spleen samples, maximum up regulation was found in T5 (8.06 fold) followed by T9 and minimum up regulation was found in T2 (1.53 fold) group. In bursa of fabricius maximum up regulation was found in T9 (13.57 fold) and minimum up regulation was found in T8 (1.39 fold) group. Up-regulation of gene expression ChTLR2b in bursa of fabricius and spleen indicates beneficial effect of organic trace minerals in potentiation of immune system in broilers.

**Acknowledgements**
The authors would like to thanks to Madhya Pradesh Council of Science Technology, Bhopal (M.P) for providing financial support to conduct the above research work.

**References**
1. Echeverry H, Yitbarek A, Munyaka P, Alizadeh M, Cleaver A, Camelo-Jaimes G et al. Organic trace mineral supplementation enhances local and systemic innate immune responses and modulates oxidative stress in broiler chickens. Poultry Science. 2016; 95:518-527.
2. Hirayama D, Lida T, Nakase H. The phagocytic function of macrophage-enforcing innate immunity and tissue homeostasis. International Journal of Molecular Science. 2018; 19(1):92-96.
3. Jaiswal SK, Raza M, Uniyal S, Chaturvedani A, Sahu V, Dilliwar L. Heat stress and its relation with expression of heat shock proteins in poultry. International Journal of Science, Environment and Technology. 2017; 6(1):159-166.
4. Kaiser P, Staheli P. Avian Cytokines and Chemokines. In: Avian Immunology. 2nd Edn, Elsevier, Boston, 2014, 89-204.
5. Kawasaki T, Kawai T. Toll-like receptor signaling pathways. Front Immunology. 2014; 25(5):461.
6. Leeson S, Caston L. Using minimal supplements of trace minerals as a method of reducing trace mineral content of poultry manure. Animal Feed Science Technology. 2008; 142(3):339-347.
7. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real time quantitative PCR. Methods. 2001; 25:402-408.
8. NRC. Nutrient Requirement of Poultry (9th Rev. Edn.). National Research Council, National Academy Press, Washington, DC, 1994, 20418.
9. Patel P, Mishra A, Singh AP, Jain AK, Kumari M, Sheikh AA. Supplementation of inorganic and organic forms of zinc, selenium and chromium on immune...
responses in broilers. International Journal of Chemical Studied. 2019; 7(6):935-942.
10. Virden WS, Yeatman JB, Barbe SJ, Willeford KO, WTL, Fakler TM et al. Immune system and cardiac functions of progeny chicks from dams fed diets differing in zinc and manganese level and source. Poultry Science. 2004; 83:344-351.
11. Yitbarek A, Echeverry H, Brady J, Hernandez-Doria J, Camelo-Jaimes G, Sharif S et al. Innate immune response to yeast-derived carbohydrates in broiler chickens fed organic diets and challenged with clostridium perfringens. Poultry Science. 2012; 91(5):1105-12.