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Effect of Supplemental Organic Zn and Mn on Broiler Performance, Bone Measures, Tissue Mineral Uptake and Immune Response at 35 Days of Age

G. Shyam Sunder, Ch. Vijay Kumar, A.K. Panda, M.V.L.N. Raju and S.V. Rama Rao
Project Directorate on Poultry, Rajendranagar, Hyderabad-30, India

Corresponding Author: A.K. Panda, Project Directorate on Poultry, Rajendranagar, Hyderabad-30, AP, India Tel: 91-40-24015651 Fax: 91-40-24017002

ABSTRACT

Supplementation of organic trace minerals is gaining importance in broiler chickens due to higher mineral bioavailability. Among the trace minerals, zinc (Zn) and manganese (Mn) are of particular interest, because they are associated with growth, skeletal soundness and immune response. Therefore, organic Zn (40, 80 or 160 ppm) and Mn (60, 120 or 240 ppm) at variable levels were tested in a factorial pattern to examine the level effects and their interactions on broiler growth, bone morphometry, mineral tissue uptake and immune response at 35 days of age. A total of 486 commercial Cobb broiler chicks was equally distributed to 9 dietary groups with 9 replicates of 6 birds each in stainless-steel battery brooders. Feed and water were offered *ad libitum* to all chicks. The results indicated that body weight gain, feed conversion efficiency, leg scores, tibia weight and tibia strength were not influenced by the supplemental levels of Zn and Mn and their interactions at 35 days of age. Bone mineralization and accumulation of Zn and Mn in tibia were significantly ($p \leq 0.05$) higher with Zn supplementation in diets at 80 ppm level, while, the Mn deposition was enhanced linearly (10.47-16.36 ppm) with progressive increase of Mn level in diets. However, Mn at 60 ppm complimented Zn retention significantly ($p \leq 0.01$). The interaction between Zn and Mn at 80 and 60 ppm, respectively supported their retention ($p \leq 0.01$) in hepatic tissue. The humoral immune response was significantly ($p \leq 0.05$) higher with Zn and Mn at 80 and 60 ppm levels, respectively. In conclusion, the results suggest that the combination of organic Zn and Mn at 80:60 ppm was synergistic for enhanced bone mineralization, mineral uptake by tissues and immune response, though the growth performance and bone morphmetry remained unchanged due to variable Zn and Mn levels and their combinations in broiler chicken at 35 days of age.

Key words: Organic zinc, manganese, performance, bone morphometry, mineral retention, immune response, broiler chicken

INTRODUCTION

Supplementation of zinc (Zn) and manganese (Mn) in broiler diets is of particular interest because they function predominantly as catalysts in many enzyme and hormone systems that are associated with growth, skeletal soundness and immune response (McDowell, 1992). Supplementation of Zn and Mn at 40 and 60 ppm, respectively was recommended in broiler diets by National Research Council (1994), considering growth as the primary response criterion. However, modern broiler chickens have undergone considerable genetic transformation

necessitating re-evaluation of mineral requirements in the context of higher growth rate, skeletal demands and immune response. Each of these criteria responded differently to Zn and Mn levels in diets, suggesting the existence of variability in mineral requirements for individual parameters (Wedekind *et al.*, 1992; Sunder *et al.*, 2006, 2011; Huang *et al.*, 2007). Equally important is the source of minerals and mineral interactions that determine the mineral bioavailability and mineral excretion (Ao *et al.*, 2009; Sunder *et al.*, 2011).

Conventionally, inorganic minerals (as oxides and sulfates) are used in chicken diets, because they are cost-effective and readily available, but are relatively inferior to organic minerals due to poor bioavailability (Virden *et al.*, 2004). In the gastro-intestinal tract, the inorganic minerals chelate with phytic acid complex and reduce their rate of absorption and consequently affect the tissue uptake of minerals (Linares *et al.*, 2007). Inorganic minerals were found to compete with each other for binding ligands and for the uptake sites in gut mucosa, reducing their absorption (Santon *et al.*, 2002). However, the extent of mineral absorption varies significantly with the interaction between the minerals which could either be synergistic (Zn and Mn) or antagonistic (Zn and Cu) based on their compatibilities (Gonzalez *et al.*, 2005; Ao *et al.*, 2009; Sunder *et al.*, 2011). In contrast, organic minerals complexed with amino-acids are devoid of free divalent cations for chelation in the intestinal lumen with phytic acid (Kidd *et al.*, 1996) and hence, they are differently metabolized facilitating enhanced absorption (Burrell *et al.*, 2004). It is in this context that organic minerals could be advantageously incorporated in diets at lower levels than the inorganic sources for realizing higher mineral bio availability and lower excretion to address the environmental concerns (Zhu *et al.*, 1998; Nollet *et al.*, 2008). Therefore, the present study aimed at evaluating the effect of organic Zn and Mn at variable levels and their interactions on broiler performance, bone morphometry, mineral bio availability and immune response, up to 35 days of age.

MATERIALS AND METHODS

Livestock, experimental diets and management: A basal diet was formulated with corn-soybean meal for starters (0-21 days) and similarly for finishers (22-35 days) with no supplemental Zn or Mn (Table 1). Commercial organic Zn and Mn was procured with mineral concentration at 10% level. The commercial organic Zn was supplemented at 40, 80 or 160 g 100 kg^{-1} feed to maintain Zn concentration at 40, 80 or 160 ppm in diets. Commercial organic Mn was similarly added to the basal diet at 60,120 or 160 g 100 kg^{-1} for maintaining Mn concentration at 60, 120 or 240 ppm. The combination of mineral levels was used in a 3×3 factorial pattern resulting in 9 combinations. Initially, all chicks were offered the basal diet from 0 to 5 days of age to deplete the mineral reserves in chicks. From 6-35 days of age, the experimental diets were offered to 9 different groups. The effects of supplemental Zn and Mn levels were evaluated independently and in combination by recording the growth performance, bone parameters, tissue mineral uptake (tibia and liver) and immune response in broiler chicken at 35 days of age.

A total of 486 Cobb broiler chicks of either sex were utilized for the feeding trial. They were individually wing banded, randomly distributed to 9 test groups with 9 replicates of 6 chicks each and housed in three-tiered stainless steel battery brooders. Each tier had two sub-units (60 cm×75 cm×45 cm) to constitute 6 cells per unit. Incandescent lights were used to brood the chicks at 34±1°C during the initial 7 days, which was gradually reduced to 26±1°C by 21 days of age and complete withdrawn thereafter. The chicks were vaccinated against Marek's disease, Newcastle disease and Infectious bursa disease as per the standard vaccination schedule. The experimental protocol was approved by the Institute Animal Ethics Committee.

Table 1: Composition of starter and finisher basal diets (kg 100 kg^{-1})

Ingredient	Starter diet (0-21 days)	Finisher diet (22-35 days)
Yellow maize	56.438	59.000
Soybean meal	38.000	33.500
Veg.oil	1.000	3.000
Di-calcium phosphate	1.500	1.500
Oyster shell	1.800	1.800
Common salt	0.500	0.440
DL -Methionine	0.180	0.180
Choline chloride (50%)	0.260	0.260
[1]Vit. AB_2D_3K	0.015	0.015
[1]Vit. B mix	0.025	0.025
[a]Coccidiostat	0.050	0.050
[b]Toxin binder	0.100	0.100
[c]Antibiotic	0.050	0.050
[2]$FeSO_4$(g)	42.00	42.0
$CuSO_4$ (g)	3.00	3.00
Na_2SeO_3 (g)	0.60	0.60
Org Mn (g)	----	-----
Org Zn (g)	----	-----
Nutrient composition		
[3]ME (kcal kg^{-1})	2845	2987
[4]Protein (%)	21.80	20.10
[4]Calcium (%)	1.140	1.120
[4]Available P (%)	0.570	0.550

[1]Supplies per kg diet: Vitamin A, 16,500 IU, vitamin D_3, 3150 ICU, vitamin K,2 mg; Thiamin, 1.2 mg; Riboflavin 10 mg; Vitamin B_6, 2.4 mg; Vitamin B_{12},12 µg, Niacin, 18 mg; Pantothenic acid, 12 mg, [2]Fe, 60 mg; Cu, 10 mg; I, 1.2 mg; Se 0.15 ppm. Inorganic salts of Analytical Reagent grade were used, Commercial organic Zn and Mn was added appropriately to provide 40, 80 or 160 ppm and 60, 120 or 240 ppm, respectively to the basal diet by replacing corn by weight, [3]Calculated values, [4]Analyzed values, [a]Cocciostat: Veldot (dinitolmide); [b]Toxin binder: Bio-bantox (phyliousilicate); [c] Anbiotic: V-Fur 200 (Furazolidone), Venky's Pvt Ltd, India

Growth, feed intake and feed efficiency: Broiler chicks were weighed individually, while the feed intake was measured in groups, replicate wise. The feed efficiency (FCR) was calculated as the ratio between feed consumption and weight gain at 35 days of age. The hock joints were scored on the basis of severity of leg abnormalities on a scale 1 to 5 in an ascending order (Waston *et al.*, 1970).

Morphometry of tibia and its mineral composition: From each dietary group 8 broilers were randomly selected and sacrificed by cervical dislocation on the 36th day. The right and left tibia from each bird were pooled group-wise, pressure cooked for 1 hour; cleared the attached muscle and cartilage, washed with distilled water and oven dried. The left tibiae were weighed and ashed in muffle furnace at 600±5°C for 4 h. Total ash was estimated on percent weight basis. From each bone, 0.2 g ash was dissolved in 5 mL of 50% HCl and the mineral extract was filtered into a volumetric flask. The blank and digested samples were filtered and diluted to the required volume to estimate the concentration of Zn, Mn and Cu in bone using atomic absorption spectrophotometer (Perkin Elmer, Analyst 400, Perkin Elmer, Life and Analytical Services, Shelton, CT 06484-4794, USA). The right tibia was used for measuring the breaking strength (EZ test, Shemadzu, Japan).

Mineral composition of hepatic tissue: Whole liver from 8 birds per dietary group were collected from the same birds that were sacrificed on the 36th day and preserved in deep freeze (-4°C) for subsequent processing. They were thawed to room temperature, oven dried at 100°C for 24 h and finely ground for mineral analysis. Approximately 0.5 g of ground sample was pre-digested with 5 mL of concentrated HNO_3 and continued digestion for 1 h at 120°C using Tecator system-2000. The contents were further digested with 30% H_2O_2 at 200°C for 45 min. A blank and digested sample was filtered, diluted to the required volume for estimating the concentration of Zn, Mn and Cu in hepatic tissue following the manual of Analyst 400.

Immune response: The humoral immune response was determined in broiler chicken using Sheep Red Blood Cells (SRBC), a non-pathogenic antigen. From each dietary group, 8 broilers were injected with 0.1 mL of 0.5% SRBC suspension into the brachial vein on the 29th day and blood samples were collected on the 5th day of post-inoculation. Subsequently, micro-haemagglutination activity of serum was estimated and the antibody titers (log_2) were measured following the standard procedure (Wegmann and Smithies, 1966).

The cell-mediated immunity was assessed (8 birds/dietary group) by measuring the hypersensitivity response of cutaneous basophils (CBH) to phytohaemagglutinin-P (PHA-P). On the 33rd day, the web thickness between 3 and 4 inter-digital space of left and right feet was measured using micrometer. The web of right foot was injected with 100 μg of PHA-P suspended in 0.1 mL of Phosphate Buffer Saline (PBS) and the left foot (control) with 0.1 mL of PBS. The web swelling was measured in both feet after 24 h of injection. The cell-mediated response was determined by subtracting the skin thickness of first measurement from the second and the values of left from right foot (Corrier and DeLoach, 1990).

Statistical analysis: The data were subjected to 3×3 factorial analyses by conducting two-way ANOVA (Snedecor and Cochran, 1989). The supplemental levels of Zn and Mn were considered as the independent factors to evaluate the level effects on different parameters. The combinations of Zn and Mn levels (3×3) were considered for estimating the interaction effects. The mean values of Zn and Mn levels and their interactions were compared using Duncan multiple range test (Duncan, 1955).

RESULTS AND DISCUSSION

The present study was conducted to evaluate the response of broiler chicken to supplemental organic Zn (40, 80 or 160 ppm) and Mn (60, 120 or 240 ppm) in diets and their interactions on growth, bone morphometry, mineral uptake by tissues and immune response at 35 days of age. The feed ingredients of the basal diet contributed Zn and Mn at 23.2 and 12.5 ppm, respectively which was common to all the test diets.

Performance of broiler chicken: Supplementation of organic Zn at 40, 80 or 160 ppm and Mn at 60, 120 or 240 ppm to a corn-soybean diet did not influence the body weight gain, feed intake or Feed Conversion Ratio (FCR) in broiler chicken up to 35 days of age (Table 2). The interaction between the levels of Zn and Mn exhibited no significant effect on these parameters. Earlier studies have indicated that Zn and Mn content in corn-soybean diet often met the requirement for chick growth and that no significant change in weight gain was realized by supplemental Zn (Wedekind *et al.*, 1992; Huang *et al.*, 2007) or Mn to the basal diet (Sunder *et al.*, 2006). However,

Table 2: Effect of supplemental organic Zn and Mn on body weight gain, feed intake/weight gain (FCR), leg scores, tibia weight, tibia strength and ash content in broilers at 35 days of age

		Performance traits			Tibia measures		
Test mineral	Supplemental levels (ppm)	Weight gain (g)	FCR	Leg scores	Weight (g kg^{-1})	Strength (N)	Ash (%)
Zn	40	1453	1.69	2.71	2.50	69.0	47.7^{b}
	80	1447	1.70	2.82	2.51	65.6	49.2^{a}
	160	1465	1.68	2.82	2.51	71.2	49.6^{a}
Mn	60	1454	1.70	2.84	2.55	67.8	48.6
	120	1456	1.70	2.79	2.45	61.6	49.3
	240	1454	1.67	2.72	2.51	76.5	48.5
[1]SEM		6.31	0.007	0.03	0.03	2.82	0.29
Source of variation		[2]Significance of treatment effect					
Zn×Mn		NS	NS	NS	NS	NS	NS
Org Zn levels		NS	NS	NS	NS	NS	*
Org Mn levels		NS	NS	NS	NS	NS	NS

[1]Standard error of the mean, [2]Significance of treatment effect: NS: Not significant, *p≤0.05, **p≤0.01, [a-b] Means within a column for Zn levels having one common superscript are not significantly different

some studies have indicated that inclusion of supplemental Zn in small quantities (20-25 ppm) in diets were required for growth (Mohanna and Nys, 1999; Huang *et al*., 2007). Despite, variations in the recommendation of Zn levels for optimum growth, evidence existed to show that whenever Zn supplementation was reduced, the endogenous Zn excretion decreased to prevent the possible negative effect on growth (Emmert and Baker, 1995). In our study, the basal diet contained Zn and Mn at 23.2 and 12.5 ppm, respectively and, supplementation of organic Zn (40 ppm) and Mn (60 ppm) as per the National Research Council (1994) recommendations satisfied the requirements for growth and FCR. Increase in supplementation of both minerals by two and four folds had no effect on growth and FCR, implying that both parameters were not sensitive to mineral changes in diets (Luo *et al*., 1991; Sunder *et al*., 2011).

Tibia measures: The results on leg scores, tibia weight and tibia strength indicated that skeletal health was not influenced by supplemental organic Zn and Mn, either individually or in combination (Table 2). Lower levels of Zn and Mn at 40 and 60 ppm, respectively were as effective as the higher levels for bone parameters. In tibia, the shaft portion represents the more rigid state of bone with high mineral deposition, while the head is an active component of change and remains susceptible to variations in mineral bio-availability (Sebastian *et al*., 1996). In the current study, minimum supplemental levels of Zn and Mn at 40 and 60 ppm, respectively were adequate to maintain leg scores, implying that the mineral deposition in the shaft and head of tibia was optimum. Published data from our previous study with inorganic Zn (40-160 ppm) and Mn (60-240 ppm) also did not impact any change in tibia weight and tibia strength reconfirming that minimum supplementation of Zn and Mn was adequate for maintaining normal bone structure (Sunder *et al*., 2011). However, supplemental organic Zn at 80 ppm level exhibited higher percent ash ($p \leq 0.05$) than 40 ppm, possibly due to enhanced Ca retention in tibia as a complimentary effect of Zn on Ca (Sunder *et al*., 2008), but further increase of Zn to 160 ppm did not improve bone mineralization.

Table 3: Influence of supplemental organic Zn and Mn levels on mineral uptake (Zn, Mn and Cu) by tibia and liver tissues in broiler chicken at 35 days of age

		Mineral content in tibia (ppm)			Mineral content in liver tissue (ppm)		
Test mineral	Supplemental levels (ppm)	Zn	Mn	Cu	Zn	Mn	Cu
Zn	40	209^{b}	13.17^{b}	9.16	93.50^{b}	9.12^{b}	16.51^{b}
	80	235^{a}	14.65^{a}	8.79	94.30^{b}	9.96^{a}	17.64^{b}
	160	245^{a}	14.16^{a}	8.87	96.80^{a}	9.69^{a}	19.21^{a}
Mn	60	242^{x}	10.47^{z}	9.94^{x}	97.50^{x}	9.17^{y}	17.91
	120	237^{x}	15.14^{y}	8.77^{y}	95.30^{x}	9.06^{y}	17.24
	240	209^{y}	16.36^{x}	8.10^{z}	91.90^{y}	10.57^{x}	18.20
[1]SEM		4.79	0.36	0.15	0.58	0.14	0.34
Source of variation		[2]Significance of treatment effect					
Zn×Mn		**	**	**	**	**	NS
Organic Zn		**	**	NS	*	**	**
Organic Mn		**	**	**	**	**	NS

[1]Standard error of the mean, [2]Significance of treatment effect: NS: Not significant, *p≤0.05, **p≤0.01, $^{a-b}$ Means within a column for Zn having one common superscript are not significantly different, $^{x-z}$ Means within a column for Mn having one common superscript are not significantly different

Mineral retention in tibia: Among the three levels of Zn tested, 80 ppm significantly (p≤0.01) enhanced Zn retention and also complimented Mn uptake by tibia compared to 40 ppm, while no significant difference was observed between 80 and 160 ppm levels (Table 3). Increasing organic Zn in diets from 40 to 160 ppm did not affect Cu retention in bone, implying that organic Zn neither enhanced nor depressed Cu content in tibia. Bone was found to be a reliable measure for evaluating the Zn and Mn requirements in chicks (Wedekind *et al.*, 1992; Huang *et al.*, 2007), because it remained as a functional reserve for minerals and mobilized Zn and Mn whenever required (Sandoval *et al.*, 1998). However, in our study a linear increase in Zn accumulation did not occur when dietary supplementation was increased from 80 to 160 ppm, implying that a plateau in Zn concentration of tibia was achieved with its inclusion in diets at 80 ppm level. Similar to our findings, Huang *et al.* (2007) realized maximum Zn concentration in the tibia of broiler chicken at 21 days of age, when Zn was supplemented in diet at 84 ppm level, which was twice the NRC recommended level (National Research Council, 1994).

In contrast to Zn, accumulation of Mn in bone increased linearly (p≤0.01) with incremental increase of Mn in diets from 60 to 240 ppm and antagonized (p≤0.01) the retention of Cu (Table 3). In our earlier study with varied levels of inorganic Mn from 0 to 3200 ppm also exhibited a linear relationship between its level of supplementation and retention in bone (Sunder *et al.*, 2006). The same study further revealed the complimentary effect of inorganic Mn (400 and 800 ppm) on Zn retention in bone. In the current study, organic Mn complimented Zn retention in bone at much lower levels (60 or 120 ppm) of supplementation, possibly due to the organic nature of both minerals. Chelation of minerals with amino acids was found to be useful in protecting them from binding with ligands and enhance absorption. This perhaps facilitated synergism between both minerals even at lower levels of supplementation (Smith *et al.*, 1995). However, Mn supplementation at 240 ppm significantly (p≤0.01) reduced Cu concentration in bone, irrespective of the Zn level in diets, indicating antagonism between the two minerals. In our earlier study, the antagonism between inorganic Mn and Cu was observed, which was believed to be due to their competition at the absorption sites in small intestines (Sunder *et al.*, 2011), but in the present

study, even organic Mn antagonized Cu retention in bone, despite the mode of Mn absorption being different. It was possible that the combinations of organic Mn and Zn induced the production of metallothionein in the intestinal mucosa, which perhaps had higher binding affinity for Cu, restricting its retention in tibia (Ao *et al.*, 2009).

The combination of organic Zn and Mn at 160:60 ppm, respectively supported higher Zn retention (271 ppm) in tibia, which was statistically not different from 80:60 ppm, suggesting that higher Zn uptake was facilitated by lower Mn level. Similarly, Zn at 40 or 80 ppm levels enhanced Mn retention in bone, suggesting the evidence of mutual complimentary effect of Zn and Mn at lower levels of supplementation in diets. However, the interaction between Zn (160 ppm) and Mn (240 ppm) at higher levels of inclusion significantly ($p \leq 0.01$) affected the retention of Zn (203 ppm) and Mn (14.15 ppm) in bone compared to lower levels of supplementation. Since, organic Zn and Mn used in the present study were in organic form, perhaps chelation of minerals with phytic acid complex of corn-soybean diet was prevented and the competition at absorption sites in intestinal lumen was minimum (Kidd *et al.*, 1996), resulting in higher mineral bioavailability compared to inorganic sources (Burrell *et al.*, 2004). Organic Zn at 160 ppm level being higher than that required perhaps induced synthesis of intestinal metallothionein to bind excess Zn and other divalent cations, making them less available for absorption (Sebastian *et al.*, 1996). Therefore, the complimentary effect between organic Zn and Mn was more pronounced even at lower levels of inclusion.

Mineral concentration in hepatic tissue: Accumulation of Zn and Mn in hepatic tissue was related to their levels of supplementation in diets (Table 3). Organic Zn at 160 ppm level produced significantly ($p \leq 0.01$) higher Zn concentration (96.8 ppm) in hepatic tissue than 40 or 80 ppm. However, Zn at 80 and 160 ppm enhanced ($p \leq 0.01$) Mn and Cu retention in hepatic tissue. Similar to Zn, Mn retention (10.57 ppm) was significantly ($p \leq 0.01$) higher in liver tissue when supplemented in diet at 240 ppm compared to 60 or 120 ppm (9.06-9.17 ppm). However, lower levels of Mn at 60 and 120 ppm complimented ($p \leq 0.01$) Zn uptake by hepatic tissue, while 240 ppm considerably reduced the same, indicating that interaction between both minerals was effective at lower level of Mn inclusion (60 ppm). However, Cu concentration in hepatic tissue was not altered by dietary Mn levels. Our earlier results with similar levels of inorganic Zn and Mn (Sunder *et al.*, 2011) and those of Henry *et al.* (1987) and Sandoval *et al.* (1998) reported linear accumulation of Zn and Mn in liver tissues when both minerals were supplemented at graded levels in diets. However, in the current study Zn and Mn retention in hepatic tissue increased significantly ($p \leq 0.01$) only at 160 and 240 ppm, respectively and not at lower levels. Relatively, Zn deposition in liver tissue was lesser than in bone. Zn accumulation in bone was higher by 2.1 to 2.7 times than in liver, indicating that tibia was more responsive to mineral accumulation than liver (Emmert and Baker, 1995) and higher Zn and Mn supplementation was required for significant increase of mineral concentration in hepatic tissue.

The interaction between the levels of organic Zn and Mn at 160:60 ppm, respectively supported higher ($p \leq 0.01$) Zn retention (99.86 ppm) in hepatic tissue, though not different from the lowest levels of Zn and Mn (40: 60 ppm). Zhu *et al.* (1998) compared organic and inorganic sources of Mn and found higher Mn retention (26%) in hepatic tissue with organic source, which perhaps complimented Zn retention in our study. However, Mn and Zn at 240 and 160 ppm, respectively enhanced Mn retention ($p \leq 0.01$) in liver compared to other combinations, implying that Zn exhibited complimentary effect on Mn retention at higher level of inclusion, but not the vice-versa.

Organic Mn at 240 ppm in combination with all levels of Zn reduced ($p \leq 0.01$) the concentration of Zn in hepatic tissue. It was possible that excess Mn accumulated in liver got excreted through biliary and intestinal pathways to establish Mn homeostasis and in the process affected Zn retention. Further, organic Zn at 160 ppm with all levels of Mn supported higher Cu accumulation ($p \leq 0.01$) in liver tissue, despite some inconsistencies. This was not in line with earlier studies which reported antagonism between inorganic Zn and Cu (Gonzalez *et al*., 2005; Ao *et al*., 2009). In the present trial, both Zn and Mn being in the organic form perhaps did not interfere with Cu absorption in the duodenal mucosa, resulting in higher Cu accumulation in hepatic tissue (Santon *et al*., 2002).

Immune response: The humoral immune response to inoculation of sheep RBC (SRBC), measured as antibody titers (log_2) was significantly ($p \leq 0.01$) higher with supplemental organic Zn at 80 ppm level compared to 40 ppm and not different from 160 ppm in broiler chicken at 35 days of age (Table 4). Relatively, antibody titers were higher with 80 ppm level than 160 ppm, indicating that Zn supplemented twice the level recommended by National Research Council (1994) was adequate for humoral immune response. Organic Zn at 80 ppm level supported the Cell Mediated Immune (CMI) response to phyto-haemagglutinin (PHA-P) better than higher or lower Zn levels, though non-significantly. Similarly, the weight of bursa and spleen was more with Zn at 80 ppm in diet, but the increase was non-significant. Higher antibody titers ($p \leq 0.05$) were also recorded with organic Mn at 60 ppm level in diet compared to 120 ppm, but it did not influence the CMI response or bursa weight. However, spleen was heavier ($p \leq 0.01$) with 120 ppm compared to 60 or 240 ppm. The interaction between organic Zn and Mn caused significant ($p \leq 0.05$) variation in antibody titers (4.92-7.42 log_2), the highest titers were due to the combination of Zn and Mn at 80:240 ppm, respectively which was not different from that of 80:60 or 160:120 ppm. The CMI response (0.58-0.86) and bursa weight (0.653-0.759 g kg^{-1}) varied non-significantly, but the spleen weight was influenced by Mn at 120 ppm with all combinations of Zn.

Zn plays an important role in immunomodulation by increasing the thymocyte and peripheral T-cell counts and interferon production, which was perhaps responsible for elevated humoral immune response in our study, particularly when organic Zn was supplemented at 80 ppm in diets

Table 4: Influence of supplemental organic Zn and Mn levels on humoral (antibody titers-log_2) and cell mediated response to PHA-P, weight of bursa and spleen in broilers at 35 days of age

Test mineral	Supplemental level (ppm)	Antibody titers	PHA-P response	Weight of bursa	Weight of spleen
Zn	40	5.44[b]	0.656	0.691	1.140
	80	6.72[a]	0.777	0.710	1.160
	160	6.14[a]	0.674	0.721	1.070
Mn	60	6.47[a]	0.727	0.687	0.974[z]
	120	5.69[b]	0.713	0.719	1.280[x]
	240	6.14[ab]	0.669	0.716	1.120[y]
SEM		0.31	0.030	0.019	0.024
Source of variation		[2]Significance of treatment effect			
Zn×Mn		**	NS	NS	*
Zn levels		**	NS	NS	NS
Mn levels		*	NS	NS	**

[1]Standard error of the mean, [2]Significance of treatment effect: NS: Not significant, *$p \leq 0.05$, **$p \leq 0.01$, [a-b]Means within a column for Zn having one common superscript are not significantly different, [x-z]Means within a column for Mn having one common superscript are not significantly different

compared to other levels (Kidd *et al*., 1996). Similar increases in antibody titers were also observed in our earlier trials with inorganic Zn at 80 ppm in broilers at 28 and 35 days of age (Sunder *et al*., 2008, 2011). However, in the present study, Zn requirements varied with the functional demands of the chicken, being higher for tissue mineral deposition (160 ppm) and lesser for growth (40 ppm) compared to humoral immune response (80 ppm). These results were consistent with those reported by Huang *et al*. (2007), who indicated that Zn requirements varied with the functional needs of broiler chicken. The CMI was also higher with dietary Zn at 80 ppm compared 40 or 160 ppm, though non-significantly. This may be due to higher production of interleukin-2 by Zn at 80 ppm (Kidd *et al*., 1996).

Mn was also involved in chicken immune response by interacting with the plasma membrane of neutrophils and macrophages that are responsible for immune competence (McDowell, 1992). In the present study, supplementation of organic Mn at 60 ppm significantly ($p \leq 0.05$) increased antibody titers possibly by stimulating the activity of neutrophils and macrophages. However, Mn level in diets at 120 ppm reduced the antibody titers, suggesting the relevance of Mn at lower level in triggering humoral immune response. In our previous study with similar levels of inorganic Mn, higher antibody titers were recorded with Mn at 120 ppm level (Sunder *et al*., 2011) while in the present study, a similar effect was realized with organic Mn at 60 ppm, suggesting the advantage of organic source over the inorganic source. Previous studies (Bao *et al*., 2007; Nollet *et al*., 2008) have reported that organically complexed trace minerals exhibited rapid absorption and assimilation compared to inorganic minerals, which perhaps facilitated higher humoral immune response with organic Mn at lower level (60 ppm). The combination of organic Zn and Mn at 80:60 ppm, respectively complimented each other for higher humoral immune response (6.92 $\log_2$), which was not different from higher titers (7.42 $\log_2$) realized by the combination of Zn: Mn at 80:240 ppm. Together, both minerals perhaps complimented the functional activity of superoxide dismutase, which is vital for the integrity of macrophage and heterophils that are responsible for elevating the antibody titers (Wellinghausen *et al*., 1997).

CONCLUSION

Evidently, in the present study, broiler growth performance, FCR and bone morphometry remained uninfluenced by the levels of organic Zn and Mn supplemented in diets, since all the combinations exhibited similar effect. However, organic Zn and Mn at 80 and 60 ppm, respectively expressed synergism for higher mineral bio-availability and immune response in broiler chicken up to 35 days of age, which was either equivalent or better than higher levels. Interestingly, organic Zn supplementation at all levels of inclusion exhibited no antagonism to Cu retention in bone and liver tissues, a significant departure from inorganic Zn. In contrast, organic Mn affected Cu uptake in bone, but not in hepatic tissue. It appeared that the response of broilers to variable levels of Zn and Mn varied with their functional needs, being lower for growth, moderate for immune response and higher for tissue uptake.

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