Effect of Supplemental Manganese on Mineral Uptake by Tissues and Immune Response in Broiler Chickens

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Two feeding trials were simultaneously conducted using 240 and 120 broiler chicks (Hubbard) for evaluating the mineral retention by tissues and immune competence, respectively as influenced by manganese (Mn) levels in feed. Mn was added at 0, 100, 400, 800, 1600 or 3200 ppm levels to a corn-soya basal diet, using sulphate salt. In both trials, chicks were uniformly distributed into 6 experimental groups and housed in stainless steel battery brooders. From 8–28 days of age, each experimental diet was fed *ad libitum* to one test group. Results indicated that Mn supplementation from 0–800 ppm did not influence body weight gain or feed consumption, but a significant depression in both parameters was noticed at 1600 and 3200 ppm levels. The feed conversion efficiency was however, not affected by dietary Mn levels. Excess (1600 ppm and above) or non-supplementation of Mn increased leg abnormality scores. Mn levels above 400 ppm significantly decreased Ca, P and Zn retention in tibia, which was related to higher leg scores. A linear increase of Mn content in bone, kidney, liver and pancreas was noticed with the increase of Mn in feed. The antibody titers against inoculation of sheep red blood cells or cutaneous basophil hypersensitivity to phytohaemagglutinin-P were not influenced by the dietary increases of Mn up to 800 or 1600 ppm levels, respectively. Both responses were negatively affected by increased Mn levels in feed. Heterophil to lymphocyte ratios, the indicators of stress were not altered by Mn supplementation up to 800 ppm, but higher levels produced wider ratios. In conclusion, supplementation of Mn at 100 ppm level was essential for leg health, better retention of Ca, P and Zn in bone besides optimum immune response. Mn supplementation at 100 ppm level was as efficient as higher levels (upto 800 ppm). Higher levels of Mn (1600 ppm and above) had negative effect on all parameters studied in broilers up to 28 days of age.

Key words : broiler chicken, immune response, manganese, mineral uptake, performance.

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Introduction

The role of manganese (Mn) in maintaining normal structure of tibiotarsal joint and in the functioning of metalloenzymes as a catalyst is well documented (McDowell, 1992). Mn has been identified as an important element in supporting normal immune functions in broiler chickens (Kidd, 2004), as it interacts with neutrophils and macrophages through plasma membrane cells that are involved in immune response (Hurley and Keen, 1987). In recent

times, Mn has become an element of increasing concern because of extremely rapid growth of broiler chickens, which exerts additional stress on the skeletal structure. National Research Council (NRC, 1994) recommended 60 ppm Mn in broiler chicken diet. However, under practical conditions the diets are often formulated to contain higher concentrations of Mn to overcome the possibility of its deficiency. This is because of relatively low absorption of dietary Mn in birds (Turk *et al.*, 1982). Therefore, there is a need to reassess the responses of broiler

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chicken to higher levels of Mn supplementation with particular emphasis on performance, mineral uptake by the tissues and immune competence during juvenile age.

Materials and Methods

Two feeding trials were simultaneously conducted with varied levels of Mn supplementation in diets to evaluate the mineral retention and immune response in broiler chicken from 8 to 28 days of age.

Experiment I

For the trial on mineral uptake by tissues, 240-day-old broiler (Hubbard) chicks were utilized. They were equally distributed into six experimental groups with eight replicates of five chicks each. Each replicate had uniform number of male and female chicks (3 and 2, respectively), which were reared in stainless steel battery brooders with raised wire floor. Similar managemental conditions were provided to all test groups. A basal diet of corn-soya was formulated without supplemental Mn (Table 1). In addition to the basal diet (37 ppm Mn), five test diets were prepared by incorporating Mn at 100, 400, 800, 1600 or 3200 ppm, using $\text{MnSO}_4\text{H}_2\text{O}$ (AR grade, Qualigens, India). Measured quantities of salt were added to the basal diet replacing starch by weight. Initially, from 0 to 7 days of age all chicks were maintained on basal diet for depleting the available Mn reserves in chicks. Subsequently, from 8–28 days of age one experimental diet was fed *ad libitum* to one test group.

Individual body weight of chicks and replicate-wise feed intake were recorded at weekly intervals. Feed conversion efficiency was calculated as the ratio between feed consumed and weight gained. The leg abnormality scores were recorded for each bird at the end of every week (Watson *et al.*, 1970). The degree of leg abnormality was measured by examining the hock joint. Depending upon the severity of deformity, the hock joints were assigned the scores ranging from 1 to 5. The hock joints that appeared normal were assigned score 1 ; slight swelling of tibiotarsal joint as 2 ; marked swelling of the joint as 3 ; swelling of the joint with symptoms of slipped achilles tendon as 4 and, swelling with marked degree of slipped tendon as 5. On the 29th day, 5 birds (3 males and 2 females) from each treatment were sacrificed by cervical dislocation to collect tibia, liver, kidney and pancreas from the

Table 1. **Ingredient and nutrient composition of the basal diet**

Ingredients	kg/100 kg
Yellow maize	59.55
Soybean meal	35.00
Di-calcium Phosphate	1.70
Shell grit	1.80
Common salt	0.50
Choline Chloride	0.26
DL methionine	0.26
Vitamins ¹	0.04
Trace minerals ²	0.10
Coccidiostat	0.05
Antibiotics	0.05
Starch*	0.69
Nutrient composition	
Metabolizable energy (kcal/kg) ³	2878
Crude protein (%) ⁴	22.05
Lysine (%) ³	1.19
Methionine (%) ³	0.50
Calcium (%) ⁴	1.09
Non phytate phosphorus (%) ⁴	0.45

¹Supplies per kg diet : Vitamin A, 16,500 IU ; vitamin D₃, 3150 ICU ; vitamin E, 12 mg ; vitamin K, 2 mg ; vitamin B₁, 1.2 mg ; vitamin B₂ 10 mg ; vitamin B₆, 2.4 mg ; vitamin B₁₂, 12 µg ; niacin, 18 mg ; pantothenic acid, 12 mg.

²Mn, 0 mg ; Zn, 44 mg ; Fe, 60 mg ; Cu, 10 mg ; I, 1.2 mg.

* $\text{MnSO}_4\text{H}_2\text{O}$ salt of AR grade was used at appropriate levels replacing starch by weight to obtain the test levels of Mn from 0 to 3200 ppm in diets.

³Calculated value.

⁴Analyzed value.

carcasses for estimating the mineral uptake by the tissues. The tibiae were freed from soft tissue and diaphysis, defatted in petroleum ether for 48h and dried at 100°C for 12 h. Subsequently, the bone samples were ashed at 600 + 30°C for 3h and the bone ash was estimated (AOAC, 1990). The Mn content in feed, bone ash, liver, kidney and pancreas, besides the concentration of Ca, Zn and Cu in bone were estimated using atomic absorption spectrophotometer (Perkin Elmer, Analyst 100, Operation- Manual) and P by the method of Fiske and Subbarow (1925).

Experiment II

For the second feeding trial on immune response, 120 day-old broiler (Hubbard) chicks were equally distributed into six treatment groups with four replicates of five chicks each. The feeding regimen and managemental conditions were similar to the first experiment. The effect of supplemental Mn on immune response of broiler chicks was studied by

Table 2. Effect of dietary Mn levels on body weight gain, feed consumption, feed conversion ratio and leg abnormality scores in broilers at 28 days of age

Mn levels (ppm)	Body weight gain (g)	Feed consumption (g)	Feed/weight gain	Leg abnormality score
0	735 ^a	1297 ^a	1.79	2.50 ^a
100	693 ^{ab}	1260 ^{ab}	1.82	1.50 ^{bc}
400	705 ^{ab}	1239 ^{ab}	1.76	1.16 ^c
800	695 ^{ab}	1228 ^{ab}	1.77	1.46 ^c
1600	684 ^{bc}	1214 ^{bc}	1.73	2.00 ^{ab}
3200	641 ^c	1168 ^c	1.83	2.13 ^a
SEM	6.67	10.71	0.01	0.08

^{a, b, c} Means carrying same superscript in a column do not differ significantly ($P < 0.05$).

measuring the cellular and humoral immune response besides the weight of lymphoid organs. Heterophil and lymphocyte ratios were determined to relate the stress in birds due to dietary changes.

The cellular immune response was assessed following cutaneous basophilic hypersensitivity test *in vivo* by injecting phytohemagglutinin-P (PHA-P). Five birds from each dietary group were selected on the 27th day of experimental feeding, measured the toe thickness between the 3rd and 4th interdigital space of left and right feet using a micrometer. Immediately after measurement, 100 µg of PHA-P suspended in 0.1 ml of phosphate buffer saline (PBS) was injected into the right toe and 0.1 ml of PBS into the left foot (control). The web swelling of both feet was measured after 24 h of injection. The response was determined by subtracting the skin thickness of first measurement from the second, and the values of left foot (control) from the right foot (Corrier and Deloach, 1990). For humoral immune response, two birds per replicate in each dietary group were inoculated with 0.1 ml of 1.0% suspension of sheep red blood cells (SRBC) on 24th day of experimental feeding. These birds were bled 5 days after SRBC inoculation by tapping the brachial vein, and the antibody titers ($\log_2$) were measured by microtitre haemagglutination procedure of Wegmann and Smithies (1966). On the 29th day, two birds per replicate from each dietary treatment were sacrificed and lymphoid organs (spleen and bursa) were collected, weighed and expressed as percent live weight. Blood smears from sacrificed birds were prepared and stained with May-Greenwald-Giemsa Stain (Gross and Siegel, 1983). Using oil immersion lens, heterophil and lymphocyte counts over a total of 60 cells were recorded and expressed as H/L ratios, which indicated the severity of stress in broiler

chicken due to Mn levels.

Statistical Analysis

The data were subjected to one-way analysis of variance (Snedecor and Cochran, 1989). The mean values were tested for statistical significance ($P < 0.05$) using Duncan multiple range test (Duncan, 1955).

Results

The results on body weight gain and feed consumption showed that broiler chickens were not influenced by Mn supplementation from 0 to 800 ppm during 8–28 days of age (Table 2), while the same parameters decreased with the increase of Mn supplementation to 1600 ppm or more. Body weight gain and feed consumption were the lowest in the dietary groups fed 3200 ppm Mn. However, none of the supplemental levels had any effect on the feed conversion efficiency. The leg abnormality scores were significantly high in birds fed basal diet without Mn supplementation. Inclusion of Mn to the basal diet at 100 ppm level alleviated the leg abnormalities. On increasing the Mn level in diet to 400 or 800 ppm, the leg health did not improve any further. On the other hand, Mn supplementation at 1600 and 3200 ppm levels significantly increased the leg scores equivalent to those fed basal diet without Mn supplementation.

The influence of supplemental Mn levels on tibia weight and mineralization is presented in Table 3. Dietary supplementation of Mn up to 800 ppm did not influence the tibia weight, but 1600 ppm and above caused a gradual and significant decline in tibia weight. Though, the tibia ash content varied significantly between Mn levels, no definite trend could be observed. Mn supplementation up to 400 ppm did not influence the Ca and P content in tibia

Table 3. Influence of supplemental Mn on tibia weight, percent total ash and mineral content at 28 days of age

Mn levels (ppm)	Tibia weight (g)	Total ash (%)	Ca (%)	P (%)	Mn (ppm)	Zn (ppm)	Cu (ppm)
0	2.19 ^a	51.0 ^{ab}	38.0 ^a	17.7 ^a	12.8 ^f	462 ^b	8.29 ^d
100	2.09 ^{ab}	52.0 ^a	38.2 ^a	17.8 ^a	15.3 ^e	463 ^b	8.26 ^d
400	2.18 ^a	50.6 ^{ab}	38.7 ^a	17.6 ^{ab}	18.3 ^d	484 ^a	9.97 ^c
800	1.93 ^{ab}	49.5 ^b	37.5 ^b	17.5 ^b	24.6 ^c	486 ^a	10.02 ^c
1600	1.88 ^b	50.3 ^{ab}	36.8 ^c	17.5 ^b	37.3 ^b	441 ^c	13.85 ^b
3200	1.62 ^c	50.1 ^{ab}	36.6 ^c	16.9 ^c	62.2 ^a	440 ^c	16.04 ^a
SEM	0.08	0.19	0.10	0.04	2.27	2.24	0.54

a, b, c, d, e, f Means carrying same superscript in a column do not differ significantly ($P < 0.05$).

Fig. 1. Influence of Mn supplementation on its uptake by liver, kidney and pancreas tissues (ppm).

ash, but on increasing Mn level to 800 ppm and above, their retention declined significantly in tibia. However, the Mn deposition in bone increased linearly with Mn levels in feed, and maximum retention was noticed at 3200 ppm level. The Cu uptake by bone also improved significantly with the level of Mn in feed. Similarly, the Zn retention in bone increased significantly up to 800 ppm level and declined sharply at 1600 and 3200 ppm.

The uptake of Mn by liver, kidney and pancreas tissues due to graded levels of its supplementation is depicted in Fig. 1. The content of Mn in liver, kidney and pancreatic tissues increased linearly with the concentration of Mn in the diet. Comparatively, Mn content was higher in kidneys than liver, followed by pancreas at all levels of supplementation.

The antibody titers in response to SRBC inoculation were similar among the dietary groups up to 800 ppm level, which were significantly reduced on increasing the Mn levels to 1600 ppm and above (Table 4). The CBH response to PHA-P injection

did not vary among the dietary groups up to 1600 ppm, but decreased significantly at 3200 ppm level. The heterophil to lymphocyte ratios were narrow and comparable among the dietary groups up to 800 ppm and varied widely at 1600 and 3200 ppm levels. However, the relative weights of lymphoid organs, the bursa and spleen were not influenced by any level of Mn supplementation to the diets (Table 4).

Discussion

NRC (1994) recommended supplementation of Mn at 60 ppm level for broiler chicken to realize optimum performance. In the present study, the basal diet contained 37 ppm Mn, which was adequate to support optimum body weight gain. Supplementation of Mn up to 800 ppm had no advantage in improving body weight gain or feed consumption. Henry *et al.* (1986) also observed no increase in average daily weight gain or feed intake with Mn supplementation at 40, 80 or 120 ppm to a corn-soya basal diet (35 ppm Mn). In another study conducted by Black *et al.* (1984), higher levels of

Table 4. Immune response in broiler chicken at 28 days of age as influenced by graded levels of supplemental Mn in broiler diets

Mn levels ppm	Antibody titre (log ₂)	CBH response	H/L ratio	Spleen (%)	Bursa (%)
0	5.6 ^a	0.203 ^a	0.480 ^b	0.13	0.23
100	5.2 ^a	0.206 ^a	0.474 ^b	0.13	0.22
400	5.2 ^a	0.206 ^a	0.493 ^b	0.14	0.20
800	5.2 ^a	0.213 ^a	0.517 ^b	0.14	0.21
1600	3.8 ^b	0.206 ^a	0.641 ^a	0.14	0.19
3200	3.6 ^b	0.184 ^b	0.652 ^a	0.14	0.16
SEM	0.30	0.04	0.08	0.06	0.05

^{a,b} Means carrying same superscript in a column do not differ significantly ($P < 0.05$).

CBH response- Cutaneous basophilic hypersensitivity response.

H/L ratio-Heterophil/Lymphocyte ratio.

Spleen and bursa-Percent of live weight.

Mn supplementation up to 500 ppm to the basal diet (116 ppm Mn) did not result in any reduction in growth or feed consumption. In our study, a significant decline in weight gain and feed consumption was recorded at 1600 ppm and above. It appeared that the threshold for these two parameters was possibly below 1500 ppm (Miles *et al.*, 2003). Thus, higher levels of Mn (1600 ppm and above) had negative effect on the performance of broiler chicken. Southern and Baker (1983) found that the tolerance level of Mn in broilers was below 4000 ppm and levels higher than this caused severe loss in performance. Although the maximum level of Mn supplemented in our trial was 3200 ppm, it affected the growth significantly compared to lower levels of elemental inclusion in feed.

In the present study, the hock joint abnormality scores increased significantly in groups fed the basal diet without supplemental Mn. Although the basal diet contained 37 ppm Mn, it failed to prevent leg abnormalities. Addition of Mn to the basal diet at 100 ppm level significantly alleviated the leg problems. It could be inferred that though the basal diet without supplemental Mn supported optimum growth, the same was not adequate to maintain skeletal integrity. Therefore, Mn requirement for skeletal structure was higher than that for growth. Mn is an activator of glycotransferases that are essential for syntheses of polysaccharides and glycoprotein, the precursors for development of organic matrix of bone (Underwood, 1981). Deficiency of Mn might have affected the structural components of cartilage resulting in higher leg scores. Increasing the Mn supplementation to 400 or 800 ppm had no added

advantage compared to 100 ppm. On the contrary, Mn levels at 1600 ppm or above caused more leg abnormalities. In the current study, Mn levels above 800 ppm reduced Ca and P retention in bone, indicating the presence of antagonistic effects among the elements. The leg abnormalities in birds fed higher Mn levels could also be attributed to decreased retention of Ca and P in tibia (Wedekind *et al.*, 1991).

The tissue uptake of Mn measured on the basis its deposition in bone revealed the presence of a linear relationship between Mn intake and its retention. It was observed that the birds fed no supplemental Mn retained 12.83 ppm Mn in tibia compared to 62.2 ppm in those fed 3200 ppm diet. Nevertheless, higher deposition of Mn in tibia could neither alter its weight nor the percent ash content up to 800 ppm level, but lowered the tibia weight thereafter. Similar to these findings, several workers reported a linear relationship between the concentration of Mn in bone and its level in the diet (Black *et al.*, 1984 ; Southern and Baker., 1983 ; Watson *et al.*, 1970). From the current study, it was further confirmed that Mn retention was more readily apparent in tissues when fed at higher levels. Thus, the linear uptake of Mn by bone makes this tissue a useful indicator of mineral availability (Black *et al.*, 1984). It was further observed that the Mn deposition had a positive influence on Zn concentration in bone. Although the Zn level in all test diets was maintained uniformly at 44 ppm, its retention in bone increased significantly with Mn at 400 and 800 ppm, establishing a dose responsive complimentary effect on Zn (Zhu *et al.*, 1997). In contrast, Mn supple-

mentation at 1600 ppm and above reduced the Zn uptake by bone. Thus, when the ratio between Mn and Zn increased to 18 : 1, Zn uptake declined. Similarly, the Cu level in all test diets was maintained uniformly at 10 ppm, but a progressive increase of Cu retention in tibia was noticed with the level of Mn in feed. The highest retention of Cu was observed at 3200 ppm level. Thus, the linear increase in Cu retention due to upward increase of Mn clearly depicted its complementary effect on the former. The positive relationship between Mn and Cu retention in broiler chicken was also reported by Black *et al.* (1985).

In this study, the uptake of Mn by liver, kidney and pancreas increased linearly with the levels of Mn in diet. It was well documented that as the dietary level of Mn increased, the endogenous excretion and turnover of Mn also increased (Wedekind *et al.*, 1991). However, there was a wide variation in turnover rates of Mn among tissues. The higher uptake of Mn by kidneys could be attributed to higher rate of exchange and rapid excretion of Mn by kidneys than any other tissue (Wedekind *et al.*, 1991). Estimation of Mn in liver (Black *et al.*, 1984), bone (Watson *et al.*, 1970) and kidneys (Wedekind and Baker, 1990) were considered as the sensitive and objective methods of Mn retention, particularly when it was fed at higher levels (up to 4000 ppm). The linear uptake of Mn by these tissues makes it a useful measure of mineral availability than considering leg bone deformity scores alone, as the criterion. This has been further confirmed by the present study wherein the uptake of Mn by bone, liver, kidney and pancreas increased linearly with higher levels of Mn in the diets.

Though Mn plays a role in immunological functions (Hurley and Keen, 1987 ; Kidd, 2004), no significant influence could be observed either on antibody titres or CBH response up to 800 or 1600 ppm levels, respectively. Probably, the minimum levels of Mn derived from the feed ingredients of basal diet were adequate to realize optimum immune response and additional supplementation of Mn was not required. On the contrary, higher Mn levels at 1600 ppm and above had negative effect on both humoral and cell mediated immune responses. Heterophil to lymphocyte ratio is an indicator of stress in broiler chicken and Mn supplementation to the diets up to 800 ppm did not influence the H : L

ratios, but higher Mn levels produced wider ratios indicating the possibility of dietary stress. The physiological functions of any biological system are dependent on its genetic makeup, nutrition and environment. Accordingly, the physiological manifestations get altered even if one of the above factors is changed. Nutrients play important role in the functionality of immune system (Cook, 1991), as they are the decisive factors in expression of immunity. Imbalances in nutrients or extreme deviations in feeding may impair immune competence in individuals (Boa-Amponsem *et al.*, 1991). Probably higher concentration of Mn in diets created imbalances of other nutrients that are responsible for immune functions, leading to lower responses in broilers (Latshaw, 1991).

It is concluded that Mn present in the basal diet (37ppm) was adequate to support optimum growth and immunity in broiler chicken up to 28 days of age. Supplementation of Mn at 100 ppm level to the basal diet improved bone mineralization and reduced the incidences of leg abnormality. Higher levels of Mn up to 800 ppm had no added advantage. Increase of Mn addition up to 1600 or 3200 ppm level had negative impact on weight gain, feed consumption, leg scores, retention of Ca, P and Zn in bone and immune competence. It appeared from the study that supplementation of Mn at 100 ppm optimized the performance with better profiles of mineral balances and immune competence in broiler chicken.

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