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Viral Antibody Titer and Leukocyte Subset Responses to Graded Copper and Zinc in Broiler Chicks

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ABSTRACT

The aim of this study was to evaluate the effects of graded Cu and Zn on immune system. A total of 360 day-old broilers were distributed into 9 treatments with 4 replicates using CRD design (3×3 factorial). Chicks for 42 days received Cu (35, 70, 105 mg kg^{-1} diet) and Zn (40, 80, 120 mg kg^{-1} diet). On days 8, 12 and 15, chicks were vaccinated against Newcastle Disease Virus (NDV), Infectious Bronchitis Virus (IBV) and Infectious Bursal Disease (IBD), respectively. Two chicks from each pen were bled on days 6 and 12 after vaccinations and collected sera were used in Hemaglutination Inhibition Test for NDV and ELISA test for IBV and IBD. On day 42, one bird from each pen was bled for counting leukocytes. Data analysis showed no significant interaction between graded Cu and zinc. The main effect means of titers increased by Cu at 105 ppm for NDV (day 12), IBV (days 6 and 12) and IBD (days 6 and 12). Zinc at 120 ppm slightly improved titers of NDV (day 6), IBV (day 6) and IBD (day 12). Graded Cu significantly enhanced number of heterophils and ratio of heterophil to lymphocyte (H:L), but reduced number of lymphocytes ($p<0.05$). Conversely, graded Zn significantly decreased H:L ($p<0.05$), but increased lymphocytes and monocytes and decreased heterophils. In conclusion, supplementations of Cu and Zn promoted immune system in broilers.

Key words: Copper, zinc, disease resistance, leukocyte subset, broiler

INTRODUCTION

Many poultry requirements particularly vitamins and trace minerals are based on research conducted 40 years ago with birds markedly and genetically differ than today (Waldroup, 2004). The current NRC (1994) Cu and Zn requirements for broilers are 8 and 40 mg kg^{-1}, respectively. Poultry industry is trying to find appropriate alternatives for antibiotics to improve disease resistance and maintain healthy poultry products (Abdukalykova and Ruiz-Feria, 2006).

An extensive review of Cu and its effects on immune system was reported by Prohaska and Failla (1993). This element supports the cell resistance and is necessary for the maintenance of phagocytic activity of white blood cells. Some studies showed that animals fed Cu-deficient diets, the absolute number and relative percentage of T cells decreased; especially T-helper cells (Lukasewycz *et al*., 1985) and spleenic lymphocytes markedly decreased in response to T- cell and B-cell mutagens (Lukasewycz and Prohaska, 1983). Also, microbicidal activity by phagocyte cells decreased (Boyne and Arthur, 1981) and acute and delayed inflammatory response increased

(Jones, 1984). Suttle and Jones (1986) found that Cu-deficient animals showed a decrease in antibody cell response along with increased susceptibility to infection. On the other hand, Chiou *et al.* (1999) reported no significant effect of copper supplementation in the form of copper sulphate pentahydrate at 250 mg kg^{-1} on intestinal layer of digestive tract of broilers.

Zinc is a critical element for proper immune function in animals. Zinc deficiency decreased cellular immunity (Fletcher *et al.*, 1988) and interleukin production (Dowd *et al.*, 1986). Abnormal T-lymphocyte development was thought to be the primary consequence of Zn deficiency (Dardenne and Bach, 1993). Zinc is commonly added to most diet of poultry and pigs to meet the nutritional requirements for this element, because of the poor availability of Zn in plant feed ingredients that binding to phytate (Fordyce *et al.*, 1987). Virden *et al.* (2003) reported lower mortality of hens fed supplemented Zn and Mn in their diets. The requirement of nutrients including Zn for animals is usually defined as the minimum dietary concentration required for maximum performance (Sterling *et al.*, 2005). In addition, much natural feed stuffs are marginally deficient in zinc (Cao *et al.*, 2002).

Copper and Zn are vital elements and essential nutrient requirements for growth and carcass development (Schumann *et al.*, 2000; Arias and Koutsos, 2006). The amounts of Cu and Zn requirements for broilers are 8 and 40 ppm, respectively (NRC, 1994). Some studies observed that small increase in Zn consumption (above NRC recommendation) depress Cu status in experimental animals (L'Abbe and Fischer, 1984) and human (Fischer *et al.*, 1984). However, most researches on vitamins and trace minerals have been reported more than 40 years ago (Waldroup, 2004). Sahin *et al.* (2002) reported serum and liver Cu concentration decreased ($p<0.05$) upon vitamins E and A supplementation under heat stress in broilers. Therefore, the aim of present study was to evaluate the responses of viral antibody titers and number of leukocytes to different levels of Cu and Zn in broiler diets.

MATERIALS AND METHODS

Birds and treatments: This study was conducted at experimental animal house, Ferdowsi University of Mashhad (FUM), Department of Animal Sciences (July to January, 2009). Three hundred and sixty day-old (Ross 308) male broiler chicks randomly assigned to 36 pens as 9 treatment diets with 4 replicates, using a CRD design (3×3 factorial). The treatments including Cu (35, 70, 105 mg kg^{-1} diet) and Zn (40, 80, 120 mg kg^{-1} diet) were added to basal ration (NRC, 1994) with minimum amounts of 3.9 and 3.1 mg kg^{-1} Cu and 19.09 and 17.31 mg kg^{-1} Zn during starter and grower periods, respectively (Table 1). Chicks were maintained in a standard room and had free access to feed and water during 42 days.

Immunological tests

HI test: anti-body titer against newcastle disease virus: The broiler chicks were vaccinated with a drop of 10^{-3} dilution of stock solution of B1 strain of Newcastle Disease Virus (NDV) on day 8. Two randomly selected chicks were bled from a wing vein on days 6 and 12 after vaccination. The collected sera were stored at -20°C in separate sterile vials to perform the HIT assay. The Hemaglutination Inhibition Test (HIT) was set up to determine the antibody titer as $\log_2$ of the reciprocal of the last dilution (Marquardt *et al.*, 1984).

ELISA test: anti-body titers against infectious bronchitis virus and infectious bursal disease: All birds were vaccinated against Infectious Bronchitis Virus (IBV) and Infectious Bursal

Table 1: Formulated rations and ingredients of the basal starter and grower diets

Items	Starter (1-21 days)	Gower (21-42 days)
Ingredients (%)		
Corn	54.4	64.63
Soybean meal	39.01	31
Oil	2.33	1
Limestone	1.29	1.25
Dicalcium phosphate	1.85	1.2
Common salt	0.46	0.35
Mineral premix[1]	0.25	0.25
Vitamin premix[2]	0.25	0.25
DL-methionine	0.16	0.07
Nutrient composition		
ME (Kcal kg^{-1})	3018	3050
Crude protein (%)	21.65	19.06
Ether extract (%)	4.71	3.7
Crude fiber (%)	2.72	2.63
Ca (%)	1	0.83
P (%)	0.48	0.35
Na (%)	0.2	0.15
Arginine (%)	1.56	1.32
Lysine (%)	1.37	1.1
Met + cys (%)	0.9	0.72
Methionine	0.5	0.38
Zinc (mg kg^{-1})	19.09	17.31
Copper (mg kg^{-1})	3.9	3.1

[1]Copper and zinc-free mineral premix provided per kilogram of diet: Mn (from MnO), 55 mg: Fe (from $FeSO_4.7H_2O$), 96 mg: I (from Ca (IO_3) $2.H_2O$) 1.4 mg. Se 0.4 mg. [2]Provided per kilogram of diet: vitamin A (vitamin A acetate) 8,700 IU: Cholecalciferol 2,300 IU: Vitamin E (from DL-α –tocopheryl acetate), 16 IU: Menadione (from menadione dimethyl pyrimidinol), 1.50 mg: B_{12}, 0.31 mg: Riboflavin, 6.6 mg: Niacin, 28 mg: Folic acid 0.80 mg: Thiamin 3 mg: Pyridoxine 2.50 mg: Biotin 30 mg: ethoxyquin 125 mg: Calcium pentothenate 35 mg

Disease (IBD) on days 12 and 15, respectively. On days 6 and 12 after each vaccination, two birds from each pen were bled via wing vein and collected sera stored at -20°C in separate vials to perform the assays (Kidd *et al*., 2001). First, the serum samples were thawed at 22°C and diluted 500-fold (1/500) with diluents. One hundred microliter of diluted sera was added to 96-well microplates coated with either IBV or IBD (IDEXX Inc., Westbrook, ME 04092). Then, they were covered and incubated at 22°C for 30 min. The microplates were aspirated and washed with 350 μL of sterile distilled water. Then, 100 μL of conjugated solution was dispensed into the wells and incubated for 30 more min. Also 100 μL of substrate was dispensed into the wells to develop a color reaction after incubation for 15 more min at 22°C. The enzymatic reaction was ended by adding the stop solution. The plates were read on a microplate reader at 650 nm (Molecular Devices, Sunnyvale, CA 94089) to evaluate the antibody titer against IBV and IBD (Kidd *et al*., 2001).

BLC test: blood leukocyte count: On the last day of study, one bird from each pen was randomly selected to determine the number of blood leukocytes after processing blood samples as described by Lucas and Jamroz (1961).

Statistical analysis: Data generated from the experiment was carried out in a CRD design (3×3 factorial) (Steel and Torrie, 1980). The data were subjected to ANOVA according to GLM procedure of SAS software. The treatment effects were compared at significant level of $p<0.05$ using a Duncan's multiple range test (Duncan, 1955). Since we had no significant interaction between graded Cu and Zn, only the main effect means were considered for conclusion (Table 2, 3). All data were checked for normality, using Shapiri-Wilk test.

RESULTS

Anti-body titers against newcastle disease, infectious bronchitis virus and infectious bursal disease: The results revealed no significant interaction among different levels of Cu and Zn for antibody titers (Table 2). The dietary Cu at 70 and 105 ppm significantly increased antibody titer of IBD on day12 post vaccination ($p<0.05$). However, dietary Cu at 70 ppm had higher effect on IBD titer compared to level of 105 ppm. On the other hand, the antibody titers of NDV (day 12), IBV (day 12) and IBD (day 6) increased by Cu at 105 ppm in comparison with other levels. Also, the dietary Zn at 120 ppm compared to 40 ppm increased antibody titers of NDV (day 6) and IBD (day 12), but not significantly (Table 2).

Blood Leukocyte Count (BLC): Present results showed no significant interaction for leukocytes in broilers fed different levels of dietary Cu and Zn (Table 3). The main effect means of leukocyte subset indicated that graded Cu significantly enhanced the number of heterophils ($p<0.05$) and heterophil to lymphocyte (H/L) ratio ($p<0.05$), but the numbers of lymphocytes ($p<0.05$) and monocytes decreased. Conversely, the graded Zn significantly reduced H/L ratio ($p<0.05$) and the number of heterophils, but increased the numbers of monocytes and lymphocytes (Table 3).

Table 2: Main effect means of antibody titers against Newcastle (log_2), infectious bronchitis and infectious bursal (log_{10}) disease viruses in broilers fed different levels of copper and zinc

	NDV titer		IBV titer		IBD titer	
Levels	6 dpi[1]	12 dpi	6 dpi	12 dpi	6 dpi	12 dpi
Cu						
35	3.12	2.58	2.71	2.11	2.71	0.97[b]
70	2.95	2.81	2.38	1.71	2.83	1.71[a]
105	3.04	3.18	2.64	2.14	2.88	1.69[a]
SE	0.25	0.38	0.11	0.14	0.10	0.18
40	3.09	3.04	2.59	2.16	2.82	1.50
Zn						
80	2.87	2.75	2.53	1.99	2.77	1.32
120	3.16	2.77	2.60	1.81	2.82	1.54
SE	0.25	0.38	0.11	0.14	0.10	0.18
Statistical effects (p value)						
Cu	0.968	0.573	0.15	0.079	0.478	0.012
Zn	0.742	0.916	0.50	0.252	0.919	0.672
Cu×Zn	0.878	0.913	0.54	0.187	0.965	0.778
Cu (quad)[2]	0.99	0.78	0.05	0.02	0.79	0.03

Means with different superscripts differ significantly ($p<0.05$). Days post inoculation. Quadratic (Quad) responses, estimated by using orthogonal polynomial contrasts

Table 3: Main effect means of WBC subset and H/L ratio of broilers fed different levels of copper and zinc

Levels	Hetrophils	Monocytes	Lymphocytes	H/L[1]
Cu				
35	18.91[b]	3.11	78.66[a]	0.254[b]
70	20.81[b]	2.50	76.90[ab]	0.277[ab]
105	27.20[a]	2.88	69.80[b]	0.418[a]
SE	2.33	0.59	2.48	0.048
40	25.66	2.22	71.88	0.367[a]
Zn				
80	20.25	2.75	77.58	0.273[b]
120	21.16	2.36	75.83	0.307[b]
SE	2.33	0.59	2.48	0.048
Statistical effects (p-value)				
Cu	0.033	0.925	0.049	0.022
Zn	0.211	0.294	0.106	0.071
Cu× Zn	0.388	0.615	0.383	0.365
Cu (lin)[2]	0.012	0.986	0.017	0.007
Zn (lin)[2]	0.160	0.200	0.080	0.040

Means with different superscripts differ significantly ($p<0.05$). Hetrophil to lymphocyte ratio. Linear (Lin) response estimated using orthogonal polynomial contrasts

DISCUSSION

Anti-body titers against newcastle disease, infectious bronchitis virus and infectious bursal disease: It is well known that trace elements and some chemicals such as betaine in animal diets have a critical role on the modulation of immune responses (Kidd, 2004; Hamidi *et al*., 2010). Copper and Zn are vital minerals and essential nutrient requirements for growth and development (Schumann *et al*., 2000; McCall *et al*., 2000). Mulhern and Koller (1988) reported that the antibody titers were improved in animals fed dietary Cu. They also found that a severe or marginal deficiency in Cu caused a gradual reduction of immune status of mice. Consequently, our results showed that graded Cu affected significantly on increasing antibody titer of IBD on day 12 ($p<0.05$).

The current NRC (1994) copper requirement for broilers is 8 mg kg^{-1} diet. However, our findings indicated that the dietary Cu at 70 and 105 ppm had significant effect on IBD titer on day 12 after vaccination ($p<0.05$). Since the NRC recommendation is related to 40 years ago and because of replacing new sues of birds today, we need to adjust the Cu requirements in order to cope with present conditions. According to this study, the dietary Cu at 70 ppm showed higher effect on IBD titer on day 12 compared to other levels. Therefore, considering our findings related to beneficial impact of Cu on birds' immunity, higher Cu levels more than NRC recommendation is suggested. Hence, more studies should be carried out to determine the optimum level of copper.

Kidd *et al*. (1992) reported that adding 40 ppm of Zn to broiler diets increased antibody production in broilers. Conversely, other reports showed no effect at this level (Pimentel *et al*., 1991). Present results indicated that Zn at 120 ppm improved antibody titers of NDV and IBV on day 6 and IBD on day 12. In addition, Zn at 40 ppm showed higher level of antibody titers in comparison with 80 ppm level and this is in agreed with some researchers. Our latest finding could be relating to sues of animals and conditions of experiment. Another report indicated that antibody titers did not increase significantly by dietary zinc sulfate vs zinc oxide (NRC recommended) in birds (Nassiri-Moghadam and Jahanian, 2009). Therefore, it is suggested that based on previous studies and our results, adding Zn at 40 or 120 ppm should enhance immunity in broiler chicks.

Furthermore, analysis of data indicated that the highest level of Cu and Zn has more effects on antibody titers with significant effects for Cu compared to zinc. These findings suggest that adding higher level of Cu with lower level of Zn as immunomodulator in diets will improve disease resistance and immune status in broiler chicks. On the other hand, Fischer *et al.* (1984) reported that Cu absorption decreased in the response to high dietary zinc. It seems that low levels of Zn in diet can develop the role of Cu on improvement of immune system. Thus, based on our findings and other studies, adding Cu at 70-105 ppm and Zn at 40 or 120 ppm would enhance antibody titers in broilers.

Blood Leukocyte Count (BLC): Copper plays an important role in development and maintenance of the immune system (Percival, 1998). Markevieius and Dringeliene (2004) were concluded that excessive and prolonged intakes of Cu suppress the proliferation of cytotoxic NK cells in mice ($p<0.05$). Conversely, in our study, the graded Cu at 35, 70 and 105 ppm significantly enhanced number of heterophiles and H/L ($p<0.05$), but reduced number of monocytes and significantly lymphocytes ($p<0.05$). It seems that the latest cells are sensitive to induction of Cu levels. Therefore, levels of 70-105 ppm of Cu are recommended in broilers. Since in most studies mice were used as modal thus, it is difficult to interpret their results with ours. Therefore, more studies are needed to determine the optimum level of Cu with different sources on leukocyte subset in broilers.

The requirement of nutrients including Zn for animals is usually defined as the minimum dietary concentration required for maximum performance (Sterling *et al.*, 2005). Virden *et al.* (2004) observed that the relative proportion of mononuclear cells to total leukocytes increased in progeny of broiler breeders fed a zinc-amino acid complex diet. Similarly, analysis our data showed higher monocytes and lymphocytes at 80 ppm compared to 120 ppm dietary zinc. On the other hand, Cunningham-Rundles and Cunningham-Rundles (1988) reported that an inadequate intracellular level of Zn impairs lymphocyte proliferation, since the DNA synthesis is dependent on zinc. Dardenne and Bach (1993) indicated that abnormal T-lymphocyte development is the primary consequence of Zn deficiency. Similarly, Prasad (1993) observed that deoxythymidin kinas activity, a zinc-dependent enzyme, is severely altered during state of mild Zn deficiency. Our findings demonstrated that 120 ppm of dietary Zn conversely reduced number of heterophyles and H/L ratio when compared to 40 ppm level. This finding suggested that 40 ppm of Zn should be sufficient to support the immune system which is in agreed with NRC recommendation. Therefore, base on our findings and other researches adding Cu at 70-105 ppm and Zn at 40-80 ppm to broiler diets are recommended for improvement of immune system by enhancing the number of different leukocytes.

CONCLUSION

The important conclusion of this research was that, dietary Cu at 70-105 ppm and Zn at 40 and 120 ppm revealed the immune responses to different viral diseases including enhanced antibody titers and leukocyte subset in male broiler chicks. Apart from present study, further researches should be done to determine the exact levels of Cu and Zn in order to increase immunity in broiler chicks.

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