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Changes in broiler breeder hen's immunity by zinc oxide and phytase

Sharideh, H.[1]; Zhandi, M.[2*]; Zaghari, M.[2]; Akhlaghi, A.[3]; Hussaini, S. M. H.[1] and Yousefi, A. R.[4]

[1]Ph.D. Student in Animal Physiology, Department of Animal Science, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran; [2]Department of Animal Science, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran; [3]Department of Animal Science, College of Agriculture, Shiraz University, Shiraz, Iran; [4]Department of Research, Breeding and Production of Laboratory Animals, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran

[*]***Correspondence:*** *M. Zhandi, Department of Animal Science, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran. E-mail: mzhandi@ut.ac.ir*

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Summary

Background: The immune response of aged broiler breeder hens is influenced by many factors including obesity and aged lymphatic organs, but may improve by increasing the bioavailability of various nutrients such as zinc (Zn). Dietary supplementation of phytase can improve Zn availability in senescent broiler breeder hens. **Aims:** The aim of this study was to investigate the effect of supplementary zinc oxide (ZnO) and phytase in a maize-soybean meal-based diet on immune responses of broiler breeder hens. **Methods:** In a 2 × 4 factorial arrangement, a total of 128 hens were randomly assigned into eight groups. The birds received two levels of phytase (0 or 300 U/kg diet) and four levels of ZnO (30, 60, 90, and 120 mg/kg diet) for 13 successive wk (59-72 wk of age). **Results**: Results showed that phytase supplementation significantly increased immunoglobulin M (IgM), cutaneous basophil hypersensitivity (CBH) responses, total number of leukocytes, percentage of lymphocytes, and heterophil to lymphocyte ratios. The percentage of basophils and monocytes, however, decreased with phytase supplementation. Supplementation of ZnO increased anti-sheep red blood cells (SRBC) antibody titer, IgM, CBH responses, the total number of leukocytes, and the percentage of lymphocytes. Dietary supplementation of ZnO decreased the percentage of heterophil, and heterophil to lymphocyte ratio. A significant interaction effect of phytase and ZnO was found on the total number of leukocytes and percentage of lymphocytes. **Conclusion:** Dietary supplementation of ZnO (90 mg/kg diet) and phytase had some positive effects on improving immune responses in broiler breeder hens.

Key words: Hen, Immunity, Leukocyte, Phytase, Zinc

Introduction

Insufficient nutrition levels can result in inefficient immune responses to risk factors (Kidd, 2004; Wu *e al*., 2018). Immune response is affected by various nutrients such as essential amino acids calcium (Ca), magnesium (Mg), and specially zinc (Zn), which is necessary for the immune system (McDowell, 1992). In addition, Zn plays a crucial role in different body systems such as reproduction and growth processes by affecting metabolic activities such as protein and carbohydrate metabolism and enzyme activation (Underwood, 1977). On the other hand, insufficient intracellular concentrations of Zn may cause the abnormal development of T-lymphocytes (Dardenne and Bach, 1993). Besides, the suppression of DNA synthesis or cell division, which is necessary for normal organ development, is a result of Zn deficiency. The reason for this suppression is the fact that Zn is a structural component of many metalloenzymes including those involved in gene replication and transcription such as DNA and RNA polymerases (Prasad, 1993).

Phytate is found in plant-originated feeds and accounts for about two-thirds of the total phosphorus of plant-based diets (Maenz, 2001). Lower levels of phytase activity in the gastrointestinal system of monogastrits is a reason for their inability to fully utilize phytate phosphorus (Maenz and Classen, 1998). Furthermore, due to the structure and reactive phosphate groups of phytate, it has a tendency to bind with cations such as minerals in gastrointestinal systems (Maenz, 2001). Phytate is hydrolyzed by phytase in gastrointestinal systems to produce inositol phosphates and inorganic phosphorus (Liu *et al*., 1998). In humans, inositol phosphate esters is critical for the function of some cells (Menniti *et al*., 1993; Shears, 1996) and improve the immune system's functionality through enhancing the immunocyte activity (Shamsuddin, 2002). It has been shown that phytase can ameliorate some undesirable actions of phytate in the broilers' gastrointestinal system (Cowieson *et al*., 2004). Also, by increasing nutrient uptake for immune cells in the intestine and maintaining mucin integrity, phytase has an improving effect on mucosal immunity (Cowieson and Ravindran, 2007). In addition, it is well established that dietary supplementation of phytase improves Zn availability in poultry (Sebastian *et al*., 1996). *Escherichia Coli*-derived 6-phytase is active in a wide pH range (2.5-6.0) and can

increase its effective function in the gastrointestinal system.

Since obesity and aged lymphatic organs are symptoms of broiler breeder hens after their peak production stage (Torroba and Zapata, 2003), immune responses may be affected by these complications and cause the immune system to become suboptimal. Therefore, the current study was designed to gain more insight about the effects of *E. Coli*-derived 6-phytase and ZnO on the immune responses of senescent broiler breeder hens fed with a maize-soybean meal diet after peak production periods.

Materials and Methods

Birds and experimental treatments

All procedures in the present work were approved by the Animal Care and Welfare Committee of the Department of Animal Sciences, Faculty of Agriculture and Natural Resources, University of Tehran, Karaj, Iran. One hundred twenty eight 59 wk old broiler breeder hens (Cobb 500) were selected from a commercial flock and randomly assigned to eight treatment groups (n=32/group) in a 2 × 4 factorial design with four replications and four birds each. Treatments included basal diet (2750 kcal ME and 14.4% CP; Table 1) supplemented with two levels of phytase (0 or 300 U/kg) and 4 levels of ZnO (30, 60, 90 and 120 mg/kg diet) fed for 13 successive wk (59-72 wk of age). The hens received a depletion diet (2750 kcal of AMEn/kg, 14.5% CP and 9.5 mg Zn/kg) for 2 wk before the experiment (58 and 59 wk of age) and were then fed with the experimental diet until 72 wk of age.

Anti-sheep red blood cells (SRBC) antibody assay

In order to assess humoral antibody responses, a suspension of SRBC was diluted in the phosphate buffer saline (PBS) to provide a 5% (vol/vol) suspension (Zijpp and Leenstra, 1980). At 64 and 65 wk of age, 0.2 ml of diluted blood was injected into the breast muscle of four birds per treatment. Seven days after first and second immunizations, 2 ml of blood was collected into heparinized tubes, and following centrifugation (1800 × g, 18°C) for 12 min, plasma samples were harvested and stored at -20°C until further analysis. In the present study, antibody titers against SRBC, serum concentrations of immunoglobulin G (IgG) and immunoglobulin M (IgM) were measured (Sharideh and Zaghari, 2017). Briefly, to evaluate the total antibodies of SRBC, 50 µL of PBS and 50 µL of serum were placed in the first row of wells in a 96-well V-bottom microtitration plate, and the solution was incubated for 30 min at 37°C. Then, 50 µL of PBS was added to the remaining wells to make a 2-fold serial dilution for each sample on successive rows. Eventually, 50 µL of 5% SRBC suspension was added to each well. Total antibody titers of SRBC were then recorded after 30 min of incubation at 37°C. The titers were expressed as $\log_2$ of the reciprocal of the highest dilution giving seeable agglutination. The IgG ME-resistant (MER) and IgM ME-sensitive (MES) antibody titers were then assessed using the same procedure as the total titers except that 50 µL of 0.02 molar mercaptoethanol in PBS was used instead of PBS alone. The difference between total and MER titers was considered as the MES titer.

Table 1: Composition of broiler breeder hen diets

Item	Depletion diet (%)	Experimental diet (%)
Corn	0	64.61
Corn starch	47.45	0.35
Soybean meal, 42.6% CP	11.28	16.26
Corn gluten meal, 62% CP	14.7	0.27
Alfalfa meal, 24% CF	0	8.78
Cellulose, 89% CF	0	13
Corn oil	2.26	1
Sodium bicarbonate ($NaHCO_3$)	0.28	0.22
Dicalcium phosphate	1.48	1.48
Calcium carbonate	7.78	6.52
Potassium sulfate [1] (K_2SO_4)	0.94	0
Phosphoric acid [2] (H_3PO_4)	1.09	0
Salt	0.2	0.17
Mineral premixes [3]	0.13	0.17
Vitamin premix [4]	0.02	0.02
DL-Methionine, 99%	0.3	0.11
L-Thereonine	0.3	0
L-Lysine HCl, 78%	0.26	0.08
Calculated nutrient content		
AME (kcal/kg)	2750	2750
CP (%)	14.5	14.41
Calcium (%)	3	3
Available phosphorus (%)	0.35	0.38
Sodium (%)	0.16	0.15
Lys (%)	0.64	0.65
Met (%)	0.58	0.32
Met + Cys (%)	0.81	0.35
Thr (%)	0.78	0.46
Arg (%)	0.62	0.74
Zinc (mg/kg)	9.5	30

[1] Available potassium 44.6%, [2] Available phosphorus 27.5%, [3] Provides (per kg of diet): copper ($CuSO_4 \cdot 5H_2O$), 10 mg; iodine (KI), 2 mg; iron ($FeSO_4 \cdot 7H_2O$), 50 mg; manganese ($MnSO_4 \cdot H_2O$), 120 mg; selenium (Na_2SeO_3), 0.3 mg, and [4] Provides (per kg of diet): vitamin A (retinyl acetate), 12,000 IU; cholecalciferol, 3,000 IU; vitamin E (DL-α-tocopheryl acetate), 2.5 mg; vitamin K, 0.5 mg; thiamin, 2.0 mg; riboflavin, 10 mg; D-pantothenic acid, 25 mg; niacin, 40 mg; pyridoxine, 6 mg; biotin, 0.66 mg; folic acid, 4 mg; vitamin B12, 0.035 mg. AME: Apparent metabolizable energy, CP: Crude protein, Lys: Lysine, Met: Methionine, Thr: Threonine, and Arg: Arginine

Cutaneous basophil hypersensitivity (CBH)

In order to evaluate cell-mediated immune responses, the CBH response to phytohemagglutinin-M (PHA-M) was assessed in four birds per treatment at wk 66 of age. The birds were intradermally injected 0.1 ml PHA-M (Gibco, Grand Island, NY, USA) and sterile PBS (as control) between the 2nd and 3rd digits of the right and left foot, respectively. Before and 24 h after injection, the toe web thickness was measured using a digital caliper (Akhlaghi *et al*., 2013).

Total and differential leukocyte counts

At the end of 72 wk of age, 0.5 ml blood sample was collected into ethylenediaminetetraacetic acid (EDTA)-

coated vials from the brachial vein of 4 hens per treatment. Total and differential leukocyte counts were performed using Natt-Herrick's solution (Akhlaghi *et al*., 2013) to measure changes in the percentages of basophils, monocytes, lymphocytes, heterophils, eosinophils, and the heterophil to lymphocyte (H:L) ratio following Wright's staining procedure (Burton and Guion, 1968).

Statistical analysis

Data were analyzed using the GLM procedure of SAS 9.1 (SAS, 2002). Before the analysis, data normality was tested by the UNIVARIATE procedure and the Shapiroe-Wilk test. The results were reported as mean and standard error of the mean (SEM). Tukey's test was used for multiple comparisons of the mean and statistical differences were declared at P≤0.05.

Results

Dietary ZnO (90 and 120 mg/kg diet) supplementation increased anti-SRBC titer and IgM in primary and secondary responses to SRBC compared to other treatments (30 and 60 mg/kg diet; P<0.01; Fig. 1), but supplementary phytase increased IgM only in the primary response to SRBC (P<0.05; Fig. 2). The interactive effects of phytase and ZnO on the total anti-SRBC titer, IgM and IgG in either primary or secondary responses to SRBC were not significant (Table 2).

Adding ZnO (90 and 120 mg/kg diet) and phytase to the diet increased CBH responses to PHA-M (P<0.01; Fig. 3); however, their interaction effect was not significant (Table 2). Total and differential leukocyte counts are represented in Table 3 and Figs. 4-7. The total number of leukocytes and the lymphocyte percentage were higher in Zn (P<0.01; Fig. 4 and Fig. 6) and phytase (P<0.05; Fig. 5) treated hens. Supplementary ZnO reduced heterophil percentages (P<0.01; Fig. 4) and the H:L ratio (P<0.01; Fig. 7), while dietary supplementation of phytase decreased basophil (P<0.05; Fig. 5) and monocyte (P<0.05; Fig. 5) percentages. The interaction of ZnO and phytase significantly affected the number of leukocytes and the lymphocyte percentage (Table 3).

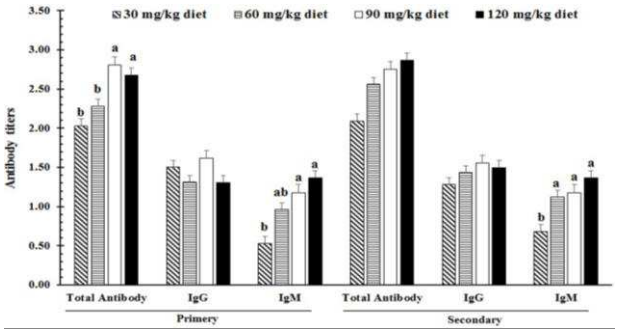

Fig. 1: The main effect of dietary zinc oxide on primary and secondary antibodies to SRBC in broiler breeders. Data are presented as mean±SEM. Different letters (a, b) represent significant differences in each parameter (P<0.05)

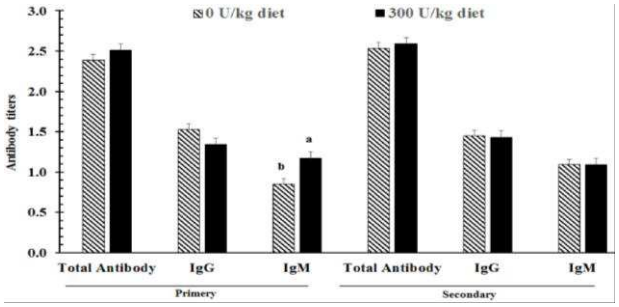

Fig. 2: The main effect of dietary phytase on primary and secondary antibodies to SRBC in broiler breeders. Data are presented as mean±SEM. Different letters (a, b) represent significant differences in each parameter (P<0.05)

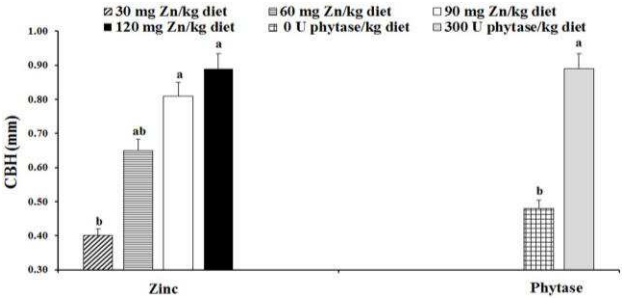

Fig. 3: The main effect of dietary zinc oxide and phytase on CBH response in broiler breeders. Data are presented as mean±SEM. Different letters (a, b) represent significant differences in each parameter (P<0.05)

Table 2: Least square means for the primary and secondary antibody to SRBC and CBH response in broiler breeders fed with diets supplemented with different levels of zinc and phytase

Treatment[1]	Factors		Primary			Secondary			CBH (mm)
	Phytase	Zinc	Total antibody	IgG	IgM	Total antibody	IgG	IgM	
1	0	30	2.00	1.62	0.37	2.06	1.43	0.62	0.34
2	300	30	2.06	1.37	0.68	2.12	1.12	0.75	0.45
3	0	60	2.18	1.50	0.68	2.50	1.37	1.12	0.47
4	300	60	2.37	1.12	1.25	2.62	1.50	1.12	0.84
5	0	90	2.75	1.50	1.25	2.75	1.50	1.25	0.51
6	300	90	2.87	1.75	1.12	2.75	1.62	1.12	1.11
7	0	120	2.62	1.50	1.12	2.87	1.50	1.37	0.61
8	300	120	2.75	1.12	1.62	2.87	1.50	1.37	1.16
SEM			0.14	0.14	0.17	0.13	0.11	0.13	0.13
P-value									
	Zinc		**	NS	**	**	NS	**	**
	Phytase		NS	NS	*	NS	NS	NS	**
	Phytase × Zinc		NS	NS	NS	NS	NS	NS	NS

[1]The birds fed with experimental diet for 13 successive wk (59-72 wk of age). NS: Non-significant,* (P<0.05), ** (P<0.01), SEM: Standard error of the mean, SRBC: Sheep red blood cells, CBH: Cutaneous basophil hypersensitivity, IgG: Immunoglobulin G, and IgM: Immunoglobulin M

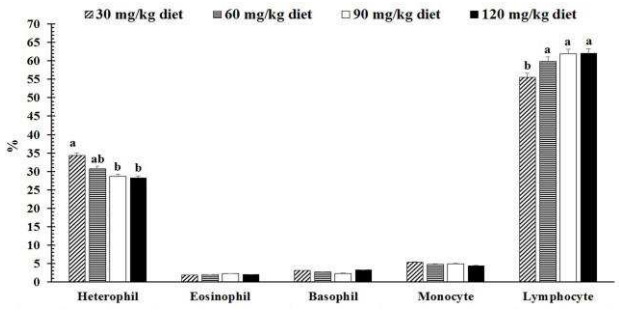

Fig. 4: The main effect of dietary zinc oxide on differential leukocyte counts in broiler breeders. Data are presented as mean±SEM. Different letters (a, b) represent significant differences in each parameter (P<0.05)

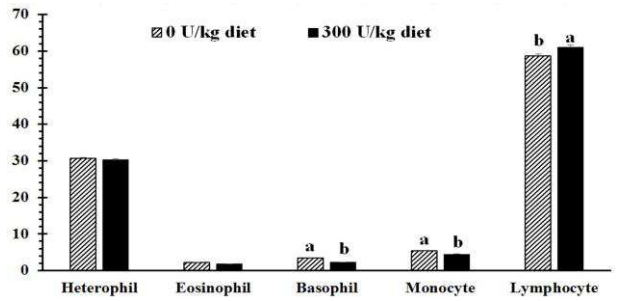

Fig. 5: The main effect of dietary phytase on differential leukocyte counts in broiler breeders. Data are presented as mean±SEM. Different letters (a, b) represent significant differences in each parameter (P<0.05)

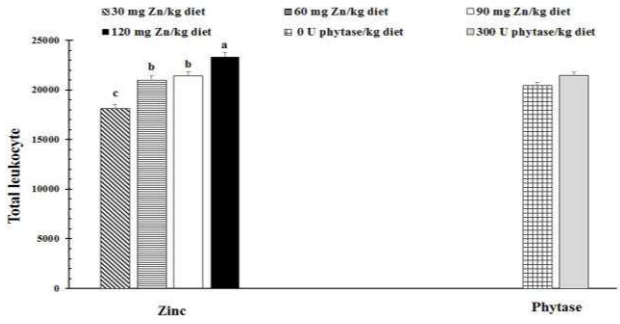

Fig. 6: The main effect of dietary zinc oxide and phytase on total leukocyte counts in broiler breeders. Data are presented as mean±SEM. Different letters (a, b) represent significant differences in each parameter (P<0.05)

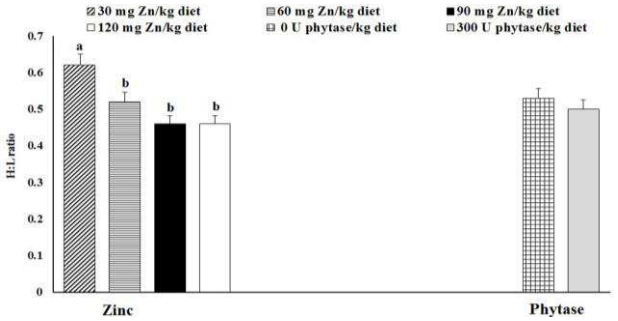

Fig. 7: The main effect of dietary zinc oxide and phytase and heterophil to lymphocyte (H:L) ratio in broiler breeders. Data are presented as mean±SEM. Different letters (a, b) represent significant differences in each parameter (P<0.05)

Discussion

In the current study, the result showed that the 90 and 120 mg ZnO/kg diets increased anti-SRBC titer and IgM in primary and secondary responses to SRBC compared to the lower doses (30 and 60 mg ZnO/kg diet), but supplementary phytase increased IgM only in the primary response to SRBC. The current results are in agreement with previous reports (Smith, 2003; Feng *et al*., 2010) showing that the dietary inclusion of Zn was associated with an increase in humoral immune responses in broilers raised under heat stress conditions. Zinc is known to have a key role in immune responses (Wellinghausen and Rink, 1998). Another important role suggested for Zn is its immunomodulatory function through two different ways including the enhancement of the number of peripheral T-cells and thymocytes, and stimulation of the interferon production (Kidd *et al*., 1996), which seems to be the case in the dietary inclusion of ZnO in the current study as well. Liu *et al*. (2008) showed that dietary Zn supplementation improved the anti-Newcastle disease virus (NDV) antibodies and the levels of intestinal secretory IgA, indicating that immune responses could be improved by dietary factors. Zyla *et al*. (2000) have reported that inclusion of phytase in diets with phosphorus deficiency increased the bursa weight in 21-d-old Hubbard broilers; the effects that may increase the proliferation B cells.

Table 3: Total and differential leukocyte count and H:L ratios in broiler breeder hens fed with diets supplemented with different levels of zinc and phytase

Treatment[1]	Factors		WBC						
	Phytase	Zinc	Total leukocyte	Heterophil (%)	Eosinophil (%)	Basophil (%)	Monocyte (%)	Lymphocyte (%)	H:L ratio
1	0	30	18235 ^{d}	33.7	2.1	3.6	5.7	54.7 ^{c}	0.61
2	300	30	17975 ^{d}	34.8	1.4	2.5	4.8	56.2 ^{bc}	0.62
3	0	60	19375 ^{cd}	32.1	2.2	3.4	5.6	56.4 ^{bc}	0.57
4	300	60	22627 ^{ac}	29.3	1.6	1.8	3.9	63.2 ^{a}	0.46
5	0	90	21625 ^{ac}	27.9	2.0	2.8	4.8	62.3 ^{a}	0.44
6	300	90	21200 ^{bc}	29.3	2.3	1.8	4.9	61.5 ^{ab}	0.48
7	0	120	22602 ^{ab}	28.8	1.9	3.6	4.7	60.7 ^{ab}	0.47
8	300	120	24112 ^{a}	27.6	2.0	2.7	4.0	63.4 ^{a}	0.43
SEM			619	1.3	0.2	0.5	0.5	1.1	0.03
P-value									
	Zinc		**	**	NS	NS	NS	**	**
	Phytase		*	NS	NS	*	*	**	NS
	Phytase × Zinc		*	NS	NS	NS	NS	*	NS

[1]The birds fed with experimental diet for 13 successive wk (59-72 wk of age). $^{a, b, c}$ Within each column, values without common superscripts are significantly different (P<0.05). NS: Non-significant, * (P<0.05), ** (P<0.01), SEM: Standard error of the mean, H:L ratio: Heterophil to lymphocyte ratio, and WBC = White blood cell

In the present study, addition of ZnO (higher doses) and phytase to the diet increased CBH responses to PHA-M, which is a sign of *in vivo* cell-mediated immune responses. It has been reported that the dietary inclusion of Zn caused higher productions of interleukin-2 which is associated with higher cell-mediated immunity (Kidd *et al*., 1996). Therefore, the increased CBH response in the present study might be due to the higher levels of Zn and phytase in the diet, which can, in turn, result in the higher production of interleukin-2.

It has also been shown that Zn has a curtailed role in the normal development of lymphocytes, and the lack of Zn may result in thymocyte depletion in the thymus which finally decreases T-cell helper peripheral activities and T-cell numbers (Kidd *et al*., 1996). Hudson *et al*. (2004) demonstrated that dietary supplementation of broiler breeder hens with 160 ppm Zn from Zn-amino acid (ZnAA) complex increased antibody titers to NDV and immune responses to phytohemagglutinin-P (PHA-P). Liu *et al*. (2008) showed that the percentages of $CD4^+$ and $CD8^+$ T-cells were enhanced, but $CD4^+$ and $CD8^+$ T-cells were not affected by the inclusion of phytase to the broilers' diet. They also showed that the dietary supplementation of phytase improved humoral and cell-mediated immune responses in broilers.

It has been reported that dietary Zn glycine increased sera concentrations of protein and calcium in broilers (Feng *et al*., 2010). Calcium plays an important role in regulating the function and signal transductions of lymphocytes, and promotes its proliferation (Imboden *et al*., 1985); effects that are likely involved in the improved cell-mediated immunity of the Zn and phytase-treated birds in the current study. Dietary supplementation of Zn (higher doses) and phytase resulted in an increase in the total number of leukocytes and lymphocyte percentages. Furthermore, the ZnO and phytase interaction significantly affected the number of leukocytes and the lymphocyte percentage. Supplementary Zn (higher doses) reduced heterophil percentages and the H:L ratio. A normal H:L ratio for hens is about 0.4, but can raise to 0.8 under severe stress (Gross and Siegel, 1983). Sunder *et al*. (2008) reported the heterophil to lymphocyte ratio to be narrow in broiler chickens fed with diets including higher levels of Zn, indicating that dietary supplementations of Zn at 40 ppm and above could lessen the stress. Also, it has been reported that decreased total numbers of lymphocytes and increased total numbers of neutrophils (the avian equivalent of heterophils) could be a result of Zn deficiency in animals (Vruwink *et al*., 1993). An acute inhibitory effect of Zn on cortisol secretion is also reported in humans (Brandao-Neto *et al*., 1990). It is well known that cortisol suppresses the immune system, which results in decreased lymphocyte reproduction. In this study, the addition of Zn and phytase to the diet might have increased the Zn uptake and the inhibitory effect of Zn on cortisol secretion.

In conclusion, based on the findings of the present study, dietary supplementation of ZnO (90 mg/kg diet) and *E. Coli*-derived 6-phytase can improve the humoral, cell-mediated and innate immunity of aged broiler breeder hens after peak production. However, further investigations are needed to clarify the mechanism of phytase and ZnO on immune function, particularly the interaction between Zn and phytase on immunity in senescent broiler breeder hens.

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