



Effects of Supplemental Zinc in a Wheat-Based Diet on Performance, Intestinal Viscosity, Immune System and Lipid Peroxidation of 21-Day Old Broiler Chickens

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Abstract

We investigated the effects of a wheat-based diet (WBD) supplemented with different levels of zinc on the performance, intestinal viscosity, immune system and lipid peroxidation of broiler chickens. A total of 240 Ross 308 day-old male broiler chicks were weighed and assigned to six dietary treatments with four replicates (floor pens) of ten birds per pen. Dietary treatments consisted of a WBD without Zn supplement in mineral premix (control), or with 20, 40, 60, 80, and 100 mg/kg of Zn in the diet. Feed intake, body weight gain, and feed conversion ratio were recorded after 21 days. On day 21, blood serum malondialdehyde concentration, intestinal digesta viscosity, and some internal organs were measured. Antibody titer against Sheep red blood cells (SRBC) were measured on days 7 and 14 after injection. For evaluation of cutaneous basophil hypersensitivity (CBH) response, on d 20, phytohemagglutinin was injected subcutaneously into toe web and 12 and 24 hrs after injection, the thickness of the web was measured. Supplementation of the WBD with 20, 40, 60, and 80 mg Zn/kg significantly improved feed conversion ratio ($P < 0.05$). Supplementation of Zn significantly decreased the relative weight of abdominal fat pad as well as jejunal viscosity ($P < 0.05$). Also, Zn supplementation (at all concentrations except 20 mg/kg) significantly decreased serum malondialdehyde concentration ($P < 0.05$). Anti-SRBC titer was significantly increased by supplementation of the WBD with 20 mg/kg Zn ($P < 0.05$). Supplementation of the WBD with 40 mg/kg Zn significantly increased CBH response ($P < 0.05$). Overall, the results of this study indicate the importance of Zn supplementation in WBD for improvement of FCR and physicochemical properties of the intestinal contents. Also, supplementation of Zn in the WBD is effective in enhancing immune system responses and antioxidative defense.

Introduction

Corn is the primary energy-dense ingredient in poultry diets and is free of antinutritional factors. Wheat is another grain with high usage in poultry diets and in some situations (e.g. availability, cost, etc.) is the primary grain in diets (Choct and Annison, 1992). However, wheat cell wall has rather high contents of non-

starch polysaccharides (NSP) with arabinoxylans as the main constituents (Scott *et al.*, 1998). Antinutritional properties such as increasing digesta viscosity, decreasing digesta passage rate, decreasing feed intake, reduction in nutrients digestion and absorption, and suppressing performance and growth rate have

been reported for NSPs (Choct and Annison, 1990; Choct and Annison, 1992; Yasar, 2003; Józefiak *et al.*, 2004). Increase in intestinal microbial population which has been related to the reduction in passage rate, is another negative effect of high NSP contents in poultry diets (Wu *et al.*, 2004). High NSP contents in poultry diets also negatively affect intestinal epithelium by decreasing villus height and subsequently, reduces absorptive surface in the small intestine (Choct and Annison, 1992).

Zinc (Zn), as an essential micronutrient for poultry, is effective in improving general and epithelial tissue health by contributing to cellular proliferation and growth (Shao *et al.*, 2014). Zinc is an essential component of many metalloenzymes which greatly affects their structural and functional properties. Because of great contribution in enzymatic functions, Zn plays important role in complex biochemical functions such as energy metabolism, proteins turnover, and nucleic acids synthesis (Flchuk and Valli, 1985). Zinc is an essential micronutrient which affects multiple aspects of the immune system (Prasad, 2008). Although NRC (1994) recommended 40 mg/kg Zn in broiler diets, high dietary NSP and phytic acid content can reduce bioavailability and absorption of dietary Zn (Lonnerdal, 2000). So, this study was conducted to evaluate effects of different levels of dietary Zn (20 to 100 mg/kg of diet) on performance, intestinal digesta

viscosity, and immune system of broiler chickens from 1 to 21 d of age.

Materials and Methods

Bird management, diets, and growth performance

240 day-old male broiler chicks (Ross 308) were weighed and randomly assigned to 24 deep litter floor pens (0.80 × 1.20 m). Dietary treatments consisted of a wheat-based diet (WBD) with no Zn supplements in mineral premix (control) or with 20, 40, 60, 80, and 100 mg/kg Zn. The WBD for the starter period (from 1 to 21 d) was formulated to meet the NRC (1994) minimum requirements (Table 1). The mineral premix used for basal diet formulation had no Zn sources. Zinc oxide (77% Zn) was used for making desired levels of Zn in dietary treatments. Broiler chickens were reared in an environmentally controlled room and had free access to feed and water. The temperature was maintained at 32°C for the first two days and then was gradually reduced to 24.5°C (at the rate of 2.5°C per week) by day 21. The light was provided continuously with 1 hr darkness in each 24 hrs cycle. Feed intake and body weight gain were recorded weekly and feed conversion ratio was calculated. Health status and mortalities were recorded daily during the experimental period. The animal experimentation was approved by Shahrekord University, Shahrekord, Iran.

Table 1. Ingredients and composition of the basal diet

Ingredients	(g/kg)
Wheat	537.5
Soybean meal (CP 44%)	365.4
Soybean oil	55.8
Limestone	13.9
Dicalcium phosphate	15.9
Salt	2.4
Sodium bicarbonate	1.7
DL-Methionine	2.4
Vitamin premix ¹	2.5
Mineral premix ² (without Zn)	2.5
<i>Chemical composition</i>	
ME (kcal/kg)	3000
CP (%)	21.56
Lys (%)	1.15
Met + Cys (%)	0.90
Ca (%)	1.0
P _a (%)	0.45
Zn (%)	0.45

¹Provides per kg of diet: all-trans-retinyl acetate, 2.72 mg; cholecalciferol, 0.05 mg; all-rac- α -tocopherol acetate, 4 mg; menadiione (menadiione sodium bisulphate), 2 mg; thiamine (thiamine mononitrate), 1.8 mg; riboflavin, 6.6 mg; Niacin, 9.8 mg; Ca-pantothenate, 29.7 mg; pyridoxine, 1.18 mg; folic acid, 1 mg; Cobalamin, 0.015 mg; D-biotin, 0.1 mg; choline chloride, 500 mg.

²Provides per kg of diet: 76 mg Mn (as MnO₂); 40 mg Fe (as FeSO₄·7H₂O); 4 mg Cu (as CuSO₄·5H₂O); 0.64 mg I (as NaI); 0.2 mg Se (as Na₂SeO₃·5H₂O).

Sample collection

On d 21, two broiler chickens from each replicate (eight broiler chickens per treatment) were randomly selected and blood samples were collected via brachial vein for malondialdehyde (MDA) measurements. These chickens were then weighed and slaughtered by cervical dislocation. The abdominal cavity was opened and the viscera were excised. Total gastrointestinal tract (from crop to the cloaca) was weighed and jejunal and ileal contents were sampled for digesta viscosity measurements. Moreover, abdominal fat pad and the liver were dissected and weighed individually. Finally, carcass weight was measured (Naderi *et al.*, 2014). Total gastrointestinal tract, abdominal fat pad, liver weights, and carcass yield are reported as g/kg of body weight.

Digesta viscosity

Collected jejunal and ileal digesta samples were kept on ice until further processing. Digesta samples were centrifuged at $3000 \times g$ for 15 min. The supernatant fraction (0.1 mL) was placed in a Brookfield DV-II+Pro cone-plate rotational viscometer (Brookfield Engineering Laboratories Inc., Stoughton, MA, USA) at 25°C and the viscosity of all samples was measured at a shear rate of 60/S (Khoramabadi *et al.*, 2014).

Lipid peroxidation assay

Serum malondialdehyde (MDA) concentration was determined as a biomarker of lipid peroxidation by the method described by Placer *et al.* (1966). Briefly, 0.5 mL of plasma, 2.5 mL Trichloroacetic acid (20%), and 1 mL thiobarbituric acid (6.7%) were mixed. The mixture was then heated on a water bath (95°C) for 30 min. After cooling, 4 mL of butanol was added and mixed, and then centrifuged at $2000 \times g$ for 10 min. Finally, the optical densities of the collected supernatants were measured spectrophotometrically at 532 nm. MDA standard was prepared by dissolving 25 μL of 1,1,3,3-tetraethoxypropane in 12.5 mL of 40% ethanol to give 8360 $\mu\text{mol/L}$ stock solution. Working solution was prepared by dissolving 50 μL of stock solution in 10 mL of 40% ethanol which resulted in MDA standard of 41.8 $\mu\text{mol/L}$. The working solution was further diluted with distilled water to yield 20.9, 10.45, 5.22, 4.86, 2.43, 1.22 and 0.61 $\mu\text{mol/L}$ MDA solutions. The absorbance of the standard MDA solutions was measured spectrophotometrically

at 532 nm and plotted against concentration to get the standard curve of MDA (Al-Fawaeir *et al.*, 2011).

Antibody-mediated immunity

In order to evaluate the effects of dietary treatments on the humoral immune response, all broiler chicks were immunized with sheep red blood cells (SRBC) antigen. For this purpose, SRBC suspension (2% V/V) was injected intramuscularly into the breast muscle (pectoralis) seven days post hatch. 7 and 14 d after injection, blood samples were collected via the brachial vein. Serum samples were then used to measure antibody titer against SRBC using a direct hemagglutination assay (Haghighi *et al.*, 2005). The highest serum dilution able to agglutinate an equal volume of SRBC suspension was registered as anti-SRBC titer and expressed as \log_2 of the reciprocal dilution factor.

Cell-mediated immunity

Cutaneous basophil hypersensitivity (CBH) response was used to evaluate cellular immunity. On d 20, two chicks from each replicate were randomly selected and phytohemagglutinin-P (PHA) solution in phosphate buffered saline (PBS) (100 $\mu\text{g}/0.1\text{mL}$) was injected subcutaneously between 3rd and 4th toe web of the right leg of each bird. To correct the response to PBS, PBS (0.1 mL) was injected subcutaneously in the same spot on the left leg of each bird. Twelve and 24 hrs after injection, the thickness of the webs in injected sites was measured using a digital micrometer. The CBH response was calculated by subtracting the thickness of the left leg toe web after PBS injection from the thickness of the right leg toe web after injection of PHA.

Statistical analysis

Data were analyzed according to a completely randomized design using the GLM (Generalized Linear Models) procedure of SAS software (SAS, 2008). Treatment means differences were determined by the new Duncan multiple range test at α value of 0.05 (Duncan, 1955).

Results

Growth performance

The results for feed intake (FI), body weight gain (BWG), and feed conversion ratio (FCR) in the different dietary treatments are shown in Table

2. Feed intake was not affected by any supplementary Zn levels in this experiment. Supplementation of the WBD with Zn up to 60 mg/kg significantly increased BWG during 1 to 7 d compared with control group ($P < 0.05$) but did not affect BWG during 1 to 14 d as well as

the entire period (1 to 21 d) of the experiment. Supplementation of the WBD with Zn improved ($P < 0.05$) FCR in a dose-dependent manner up to 80 mg/kg during all periods of the experiment (1 to 7; 1 to 14; and 1 to 21 d).

Table 2. Effects of different levels of supplemental Zn in a wheat-based diet on production performance of broiler chickens

Item	Supplemental Zn (mg/kg)						SEM	P-value
	0 (control)	20	40	60	80	100		
Feed intake, g								
1 to 7	85	91	86	89	87	86	4.2	0.91
1 to 14	427	448	417	423	413	422	19.5	0.83
1 to 21	1052	1024	1000	979	956	977	41.8	0.62
Weight gain, g								
1 to 7	68 ^b	77 ^a	75 ^a	76 ^a	74 ^{ab}	71 ^{ab}	2.1	0.06
1 to 14	271	295	278	292	280	273	9.4	0.37
1 to 21	593	611	609	617	576	563	45.6	0.47
Feed conversion ratio								
1 to 7	1.25 ^{ab}	1.18 ^c	1.14 ^c	1.17 ^{bc}	1.18 ^{bc}	1.20 ^{ab}	0.022	0.01
1 to 14	1.58 ^a	1.53 ^{ab}	1.50 ^{bc}	1.45 ^c	1.48 ^{bc}	1.55 ^{ab}	0.025	0.02
1 to 21	1.78 ^a	1.68 ^b	1.64 ^{bc}	1.59 ^c	1.63 ^b	1.74 ^a	0.024	0.01

^{a-c}Means in the same row with different superscript differ significantly ($P < 0.05$).

Digesta viscosity

Results of jejunal and ileal digesta viscosity are shown in Table 3. Supplementation of the WBD with Zn caused a significant ($P < 0.05$) reduction

in the viscosity of jejunal digesta compared to control group, but did not affect digesta viscosity in the ileum.

Table 3. Effect of different levels of supplemental Zn in a wheat-based diet on jejunal and ileal digesta viscosity in broiler chickens (centipoise)

Item	Supplemental Zn (mg/kg)						SEM	P-value
	0 (control)	20	40	60	80	100		
Jejunum	12.2 ^a	1.7 ^b	4.2 ^b	2.7 ^b	3.9 ^b	1.1 ^b	2.17	0.05
Ileum	7.1	5.9	2.8	5.1	4.6	1.4	2.57	0.83

^{a,b}Means in the same row with different superscript differ significantly ($P < 0.05$).

MDA concentration

Effects of different levels of supplemental Zn in WBD on blood serum MDA concentration are shown in Table 4. Supplementation of the WBD

with Zn (at all concentrations except for 20 mg/kg) significantly decreased serum MDA concentration compared to control group ($P < 0.05$).

Table 4. Effect of different levels of supplemental Zn in a wheat-based diet on serum MDA concentration in broiler chickens (measured on 21 d of age)

Item	Supplemental Zn (mg/kg)						SEM	P-value
	0 (control)	20	40	60	80	100		
MDA ($\mu\text{mol/L}$)	2.4 ^a	2.3 ^a	1.4 ^b	1.3 ^b	1.3 ^b	1.2 ^b	0.21	0.01

^{a,b}Means in the same row with different superscript differ significantly ($P < 0.05$).

Antibody-mediated immunity

Effects of different levels of supplementary Zn in WBD on antibody titer against SRBC antigen are shown in Table 5. Supplementation of the WBD

with 20 mg/kg Zn significantly increased antibody titer against SRBC 7 d post immunization ($P < 0.05$). However, this effect was not significant 14 d post immunization.

Table 5. Effect of different levels of supplemental Zn in a wheat-based diet on antibody titer against SRBC antigen in broiler chickens (\log_2 of reciprocal dilution factor)

Item	Supplemental Zn (mg/kg)						SEM	P-value
	0 (control)	20	40	60	80	100		
7 d post immunization	1.9 ^b	3.8 ^a	1.9 ^b	2.4 ^b	1.9 ^b	2.6 ^b	0.32	0.01
14 d post immunization	1.6	2.5	1.7	2.6	2.0	2.5	0.49	0.30

^{a,b}Means in the same row with different superscript differ significantly ($P < 0.05$).

Cell-mediated immunity

Supplementation of the WBD with Zn had no significant effect on CBH response 12 hrs after PHA injection (Table 6), but only

Supplementation at 60 mg/kg Zn significantly ($P < 0.05$) increased CBH response 24 hrs after PHA injection (Table 6).

Table 6. Effect of different levels of supplemental Zn in a wheat-based diet on CBH response (μm) in broiler chickens

Item	Supplemental Zn (mg/kg)						SEM	P-value
	0 (control)	20	40	60	80	100		
12 hrs post injection	664	704	595	1138	927	835	171.8	0.32
24 hrs post injection	216 ^b	458 ^{ab}	276 ^b	722 ^a	412 ^{ab}	375 ^b	105.1	0.04

^{a,b} Means in the same row with different superscript differ significantly ($P < 0.05$).

Organs weight

Effects of supplementary Zn in WBD on relative organ weights and carcass yield are shown in Table 7. Supplementation of the WBD with 20, 40, and 60 mg/kg Zn significantly decreased relative gastrointestinal weight compared to control group ($P < 0.05$). Supplemental Zn in WBD also caused a significant reduction in relative liver weight as well as the relative weight of abdominal fat pad ($P < 0.05$). The relative weight of bursa of Fabricius was not

significantly affected by any of the dietary treatments. However, the relative weight of the spleen was significantly decreased by supplementation of the WBD with 40 or 60 mg/kg Zn compared to control group ($P < 0.05$). Furthermore, supplementation of the WBD with 40 or 80 mg/kg Zn significantly increased carcass yield compared to non-supplemented diet ($P < 0.05$).

Table 7. Effect of different levels of supplemental Zn in a wheat-based diet on organs relative weight and carcass yield (g/kg of body weight) in broiler chickens

Item	Supplemental Zn (mg/kg)						SEM	P-value
	0 (control)	20	40	60	80	100		
Total gastrointestinal tract	175 ^a	141 ^b	133 ^b	146 ^b	157 ^{ab}	155 ^{ab}	5.1	0.01
Abdominal fat pad	13.1 ^a	6.5 ^b	5.7 ^b	5.3 ^b	4.8 ^b	5.2 ^b	1.3	0.03
liver	29 ^a	27 ^{ab}	22 ^b	24 ^b	26 ^{ab}	23 ^b	2.2	0.05
Carcass yield	641 ^{bc}	665 ^{ab}	678 ^a	656 ^{abc}	676 ^a	632 ^c	12.5	0.02

^{a,b,c}Means in the same row with different superscript differ significantly ($P < 0.05$).

Discussion

Consistent with our results, Liao *et al.* (2013) also reported no significant effect on FI and BWG when broiler diets were supplemented with 20 to 140 mg/kg Zn. Yogesh *et al.* (2013) supplemented broiler diets with 40 to 100 mg Zn/kg and reported no significant effects on FI and BWG. Also, supplementation of the broiler diets with 40, 120, and 200 mg/kg Zn had no significant effect on FI and BWG (Sajadifar and Miranzadeh, 2013). In contrast to our results, Askari *et al.* (2015) reported an increase in FI of

broiler chickens when wheat-based diet was supplemented with 50 and 70 mg Zn/kg. On the other hand, supplementation of the diet with high levels of Zn (1500 mg/kg Zn as ZnSO_4) had negatively affected FI and BWG of broiler chickens (Kim and Patterson, 2004). In contrast to our results, some studies found that FCR did not improve with dietary Zn supplementation in broiler chickens (Liao *et al.*, 2013; Sajadifar and Miranzadeh, 2013; Yogesh *et al.*, 2013).

High NSP contents in wheat with the majority of arabinoxylans cause high digesta

viscosity which negatively affects the morphometric characteristics of the intestinal wall (Langhout *et al.*, 1999). High intestinal viscosity has deleterious effects on intestinal epithelium such as reduction in villus height and increase in crypt depth which will lead to lower nutrient digestion and absorption (Choct and Anison, 1992). Improving effects of supplemental Zn in WBD on intestinal viscosity could be related to the effects of Zn on intestinal epithelial health (Højberg *et al.*, 2005; Hedemann *et al.*, 2006).

Malondialdehyde is a product of lipid peroxidation reactions in the body and its serum concentration is measured to assess oxidative damage (Azevedo Neto *et al.*, 2006). In contrast to our results, Hosseini-Mansoub *et al.* (2010) reported a decrease of MDA concentration in serum samples of broiler chickens fed diets enriched with 50 mg/kg Zn. As a cofactor of many antioxidative enzymes, Zn plays a key role in decreasing production of free radicals (Prasad and Kucuk, 2002). As an antioxidant agent, Zn reduces lipid peroxidation in the cell membrane (Powell, 2000; Prasad and Kucuk, 2002). Sahin and Kucuk (2003) reported that supplementation of diet with 30 mg/kg Zn decreased serum MDA content in heat-stressed laying Japanese quail. Reduction in serum MDA concentration due to dietary Zn supplementation has also been reported in some other studies (Onderci *et al.*, 2003; Sahin *et al.*, 2006).

Consistent with our result, Gajula *et al.* (2011) and Shyam Sunder *et al.* (2008) found improvement in antibody titer against SRBC in broiler chickens with dietary Zn supplementation. Yogesh *et al.* (2013) reported the maximum antibody titer against SRBC occurred at the supplementation level of 80 mg/kg Zn in broiler chickens diet. Sajadifar *et al.* (2013) also reported an increase in antibody titers against NDV vaccine by supplementation of the broiler diet with 120 or 200 mg/kg Zn. On the other hand, Askari *et al.* (2015) supplemented a broiler WBD with 50 or 70 mg Zn/kg and reported no significant effect on antibody titer against SRBC.

Cutaneous basophil hypersensitivity response has been used as an appropriate index for investigating cell-mediated immunity in poultry (Erf, 2004). In our study, supplementation of the WBD with 60 mg Zn/kg significantly increased CBH response 24 hrs after PHA injection. Similarly, Yogesh *et al.*

(2013) reported the maximum CBH response when broiler diets had been supplemented with 60 mg Zn/kg. In the research of Sajadifar *et al.* (2013), supplementation of broiler diets with 200 mg/kg Zn led to the higher CBH response compared to lower supplemental levels. In both humans and animals, Zn deficiency has led to thymic and splenic atrophy and weakened cellular and humoral immunity (Hojyo *et al.*, 2015). The role of Zn in strengthening antibody-mediated immunity has recently been investigated by Hojyo *et al.* (2015).

As mentioned earlier, the inclusion of wheat in broiler diets increases viscosity of the intestinal contents. The increase in digesta viscosity compels the broiler chicken's digestive tract to undergo adaptive changes such as increase intestinal mucosa (Olkowski *et al.*, 2005), villus width (Jin *et al.*, 1994), crypt depth (Jacobs, 1983), the number of goblet cells, and mucin production (Piel *et al.*, 2005). These kinds of adaptive processes could eventually result in an increase in relative gut weight (Yasar, 2003). In our study, supplementation of the WBD with Zn was effective in lowering digesta viscosity which led to a reduction in gastrointestinal relative weight.

Because of the low availability of biotin in WBD, broiler chickens can have fatty livers and kidney syndrome (Bryden, 1990). So, the high relative weight of the liver in our control group may be attributed to some degree of fat deposition. Zinc serves as a cofactor for many metalloenzymes, some of which are intestinal proteases (e.g. carboxypeptidase A and B) (Hopfer, 2002), and so, supplemental Zn could increase protein digestibility in poultry (Sahin *et al.*, 2009). Therefore, the decrease in abdominal fat pad relative weight that we observed with Zn supplementation may be attributed (at least in part) to better digestion/absorption/metabolism of dietary proteins. This effect could also be referred to as a cause for the reduction in relative liver weight we observed in chickens in Zn supplemented groups. Furthermore, there is some evidence that shows dietary zinc supplementation is effective in reversing alcohol-induced steatosis in mice (Kang *et al.*, 2009).

Ao *et al.* (2007) reported an increase in carcass yield of broiler chickens when the diet was supplemented with Zn. Increase in carcass yield of quails with dietary Zn supplementation has also been reported (Sahin *et al.*, 2005; Sahin *et al.*,

2006). On the other hand, Shyam Sunder *et al.* (2008) reported no effect on carcass weight of broiler chickens with dietary Zn supplementation.

Conclusion

The results of this study emphasize the importance of supplemental Zn in wheat based diets in regards to performance, immune

function, and antioxidative defense of broiler chickens. According to the results of this study, supplemental Zn could benefit digesta viscosity of broiler chickens when wheat is the main source of energy in the diet.

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