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Effect of Monovalent Copper Oxide and Potentiated Zinc Oxide on Growth Performance and Gut Morphology of Broiler Chickens Challenged with Coccidiosis

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Abstract
An experiment was conducted to evaluate the effect of copper oxide (Cu_2O) and potentiated zinc oxide (ZnO) on performance, intestinal morphology, oocyst excretion, coccidial lesion scores, and antioxidant properties in broilers during an *Eimeria* spp. challenge. A total of 288 1-day-old male broiler chickens (Ross 308) were divided into 18 treatments. Treatments included three levels of Cu (0, 15, or 150 mg/kg) from Cu_2O and three levels of Zn (0, 80, or 160 mg/kg) from potentiated ZnO which were added to the basal diet and fed to broilers with or without challenge, using a completely randomized design in a factorial arrangement for 42 days. Live body weight, feed intake, mortality, and the cause of death were recorded weekly and histomorphology of jejunum was measured at the end of the experiment. Results showed that birds fed Cu and Zn linearly decreased ($P < 0.0001$) oocyst shedding. The number of excreted oocysts was reduced eight times in broilers fed a diet containing 150 mg/kg copper from Cu_2O and 160 mg/kg zinc from potentiated ZnO, compared to the infected group without Cu and Zn supplementation ($P < 0.0001$). Microscopic features of both non-challenged and challenged broiler jejunum revealed significant improvement along with increased Cu_2O and potentiated ZnO doses. Supplementation of Cu_2O and potentiated ZnO decreased the jejunum structure damages and intestinal lesion score ($P < 0.002$). *Eimeria* caused a decrease ($P < 0.006$) in total antioxidant capacity. Superoxide dismutase increased by dietary zinc supplementation ($P < 0.05$). Results suggested that a combination of Cu_2O and potentiated ZnO could exhibit efficient anticoccidial activity.

Keywords Copper · Zinc · Broiler · Gut morphology · Coccidiosis

Introduction

Coccidiosis is an enteric contagious protozoan disease caused by *Eimeria* genus that affects poultry throughout the world, in particular in warm and humid environments. Currently, this disease is controlled via the application of antioxidants, antibiotics, and coccidiostats such as ionophores that are added directly to the broiler diets [1–3]. However, the widespread use of anticoccidial drugs has led to the development of resistant strains of *Eimeria* [4–6]. Population genetic analysis revealed that genetic polymorphisms among field isolates of *Eimeria tenella*, which is one of the highly pathogens, cause drug resistance [7].

Problems with the use of therapeutic drugs are mostly related to public concerns about drug residues in poultry products [8]. These issues have encouraged the poultry industry to find alternative ways to improve body protective systems, such as the use of plant extracts with antioxidant activity [9], vitamins A, E, and C, and trace minerals such as copper, zinc, and selenium [10–12]. Through their antioxidant properties, these substances have been shown to reduce the harmful effects of *Eimeria* by creating a high level of protection and improving the immune system of animals exposed to the disease [9]. Antioxidant compounds have been reported to reduce the severity of *Eimeria tenella* infection by improving the degree of intestinal lipid peroxidation [13]. Monovalent copper oxide (Cu_2O) at therapeutic doses (150 mg/kg of Cu) in broiler diet showed to improve performance [14], and supplementing 150 mg/kg of Cu from

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Cu_2O establishes changes in the gut microbiota by regulating the bacterial population in the ileum [15]. Also, reports suggested that excessive Cu accumulates in different organs, and free unbound copper in the blood may act as a strong oxidizing agent and cause a toxic response [16, 17].

Anticoccidial properties of ZnO were investigated [11]. It has been shown that ZnO nanoparticles are also effective against *Eimeria papillata*–induced coccidiosis. Zinc exhibits anticoccidial activity, as evident by a significant lowering in the output of *Eimeria papillata* oocysts within the feces of infected mice. Added dietary zinc decreased coccidial lesion scores in broiler chickens infected with *Eimeria brunetti* [18]. Zinc has antibacterial properties. One hypothesis is that the active oxygen species generated by zinc oxide could be the main mechanism [19]. Our recent findings demonstrated that potentiated ZnO had a significant impact on tight junction proteins and lessening the inflammatory response in the broiler breeder intestine by lowering transcript abundance of pro-inflammatory cytokines, such as TNFα and also IL6 in the serum [20, 21].

Despite of these findings, there is still a doubt whether these minerals have a direct effect against *Eimeria*. Therefore, this study aimed to evaluate the interactive effects of Cu_2O and potentiated ZnO on performance, intestinal morphology, oocyst excretion, coccidial lesion scores, and antioxidant properties in broiler chickens during the challenge with *Eimeria* spp.

Materials and Methods

All experimental methods were approved by the University Of Tehran Department Of Animal Science, Animal Welfare Committee, and it complied with the European Union guidelines for the care and use of Animals in Research [22].

Bird Management and Husbandry

The study was performed in the experimental broiler facilities of the Department of Animal Science, University of Tehran (35.8038° N, 51.00007° E). Before chick placement, the floor and walls of the building, as well as the equipment, were thoroughly disinfected and washed using commercial alkalis solutions [23]. A total of 288 1-day-old male broiler chickens (Ross 308) were purchased from a commercial hatchery and divided into 18 treatments based on the same initial bodyweight. Treatments were replicated 4 times, and each contained 4 birds per cage (75 × 72 × 35 cm dimension, and 0.54 m^2). The temperature was 33 °C from 0 to 3 days and gradually (at the rate of 2° C per week) decreased. From the fifth week onwards, the temperature reached 23 °C, which was kept until the end of the trial. The lighting program in the first 3 days included 24 h of light, provided by the compact fluorescent bulb, then 23 h until the end of the first week, and afterward gradually (during one week) reduced to 18 h of light and 6 h of dark, and this program was maintained until the end of the experiment.

Experimental Diets and Treatments

The starter, grower, and finisher diets were used from days 0 to 10, 11 to 24, and 25 to 42, respectively (Table 1). There were 18 experimental treatments organized in a factorial experimental design. Treatments included three levels of

Table 1 Composition and nutrient content of the basal diet

Item	Starter	Grower	Finisher
Ingredients, %			
Corn grain	47.25	50.33	67.48
Soybean meal 44%	42.95	39.16	28.07
Corn oil	5.40	6.35	0.99
Dicalcium phosphate	1.93	1.90	1.34
Calcium carbonate	0.97	0.89	0.79
Sodium chloride	0.37	0.37	0.36
Sodium bicarbonate	0.15	0.15	0.15
Mineral-vitamin premix[1]	0.50	0.50	0.50
DL-Methionine	0.32	0.28	0.21
L-Lysine.HCl	0.09	0.04	0.09
L-Threonine	0.07	0.03	0.02
Calculated composition[2] (g/kg)			
ME_n (Mj/kg)	12.55	12.97	12.34
Crude protein	230	215	179.8
Calcium	9.6	8.7	7.2
Available phosphorus	4.8	4.3	3.6
Sodium	2.0	2.0	2.0
Dig. lysine[3]	12.8	11.5	9.4
Dig. methionine	6.4	5.8	4.7
Dig. methionine + cystine	9.5	8.7	7.3
Dig. threonine	8.6	7.7	6.3
Analyzed composition, mg/kg			
Zinc[4]	25.68	24.76	23.37
Copper[4]	21.26	21.25	23.04

[1]Provided per kg of diet: vitamin A (retinyl acetate), 12,000 IU; vitamin D3 (cholecalciferol), 5000 IU; vitamin E (dl-a-tocopheryl acetate), 80 IU; vitamin K3 (menadione nicotinamide bisulfate), 3.2 mg; vitamin B1, 3.2 mg; vitamin B2, 8.6 mg; vitamin B6, 4.3 mg; vitamin B12, 0.017 mg; nicotinic acid, 65 mg; pantothenic acid (D-Ca pantothenate), 20 mg; biotin, 0.22 mg; folic acid, 2.2 mg; choline (choline chloride), 700 mg; Zn, 0 mg; Cu, 0 mg; Fe ($FeSO_4$), 20 mg; Mn (MnO), 120 mg; I (KI), 1.25 mg; and Se ($NaSeO_4$), 0.3 mg; antioxidant, 1 mg

[2]*ME_n*, metabolizable energy corrected for zero nitrogen retention; *Dig.*, digestible

[3]Calculated amino acid compositions is reported on a standardized ileal digestible amino acid basis (NIR spectroscopy)

[4]Zinc and copper were analyzed by ICP

Cu (0, 15, or 150 mg/kg) from Cu_2O (CoRouge, 75.4% Cu, Animine, France) and three levels of Zn (0, 80, or 160 mg/kg) from potentiated ZnO (HiZox, 75% Zn, Animine, France) added to the basal diet (Table 1), with or without challenge. The treatment arrangement was depicted in Fig. 1. The mineral and vitamin premixes included in the diet had no Cu and Zn. Diets were formulated to be isonutritives and to meet the nutrient recommendation of Ross 308 [24] except for finisher, where recommended ratios were followed. Birds had free access to feed and water throughout the experiment, and no antibiotics or growth promoters were used in the feed or water.

Performance Measurements and Sample Collection

Live body weight (BW) and feed intake (FI) were recorded weekly. Feed conversion ratio (FCR) was calculated at the end of each week. Mortality and cause of death were also recorded daily. At day 42, one bird per cage ($n = 72$) with a similar BW to the average of the cage was selected. The chickens were slaughtered and the middle part of the jejunum was immediately removed for histomorphology measurements and the entire intestinal tract was examined for the lesion. The liver weight was recorded. Subsequently, the relative weight of the liver to BW (RLW) was calculated.

Chemical Analysis

The copper and zinc content in the basal and experimental diets was determined using inductively coupled plasma optical emission spectroscopy (ICP-OES, model Optima 4300DV, PerkinElmer Inc.; Waltham, MA). The analyzed Cu and Zn contents of experimental diets were equivalent to the sum of supplemented amounts (Fig. 1) and the analyzed content in the basal diets ± 5 mg/kg. Based on 10 samples taken from the discharge of the feed mixer, the coefficient of variation (CV) of Cu and Zn content of experimental diets was lower than 3%.

Group	Cu mg/kg	Zn mg/kg	Coccidiosis Challenge
T1	0	0	-
T2	15	0	-
T3	150	0	-
T4	0	80	-
T5	15	80	-
T6	150	80	-
T7	0	160	-
T8	15	160	-
T9	150	160	-
T10	0	0	+
T11	15	0	+
T12	150	0	+
T13	0	80	+
T14	15	80	+
T15	150	80	+
T16	0	160	+
T17	15	160	+
T18	150	160	+

Fig. 1 Schematic representation of experimental treatments

Monovalent Copper Oxide and Zinc Oxide Physical Property Measurement

A sample of monovalent copper oxide and zinc oxide was taken to measure physical properties. Arithmetic mean particle size of 0.3 g, Cu_2O was 0.072 mm (SD, 0.028; CV, 38.9%) and mean particle size of ZnO was 0.167 mm (SD, 0.049; CV, 29.4%).

The ability of Cu and Zn sources to mix with feed was evaluated by the angle of repose measurement. The angle of repose is the angle (relative to the horizontal base) of the conical pile produced when a granular material is poured onto a horizontal surface. The angle of repose was measured by powder flowability tester model BEP2 (Copley Scientific Limited, Colwick Quays Business Park Private Road No. 2, Colwick Nottingham NG4 2JY UK). The angles of repose of Cu_2O and ZnO were 26° and 28° respectively. The corresponding angle of repose interpretation table (Copley Scientific, Nottingham, UK) classified the tested Cu and Zn sources in the excellent mixing ability category.

Transmission electron microscopy (TEM) image of monovalent copper oxide (Cu_2O) and potentiated zinc oxide (ZnO) is shown in Fig. 2. TEM has an unparalleled ability to provide structural and chemical information over a range of length scales down to the level of atomic dimensions. It was performed on a Tecnai G2 20 S-Twin electron microscope at an accelerating voltage of 20 kV. Specimens for TEM measurement were prepared by depositing a drop of colloid solution on a 400 mesh copper grid coated by an amorphous carbon film and evaporating the solvent in the air at room temperature [20].

Coccidiosis Challenge Procedure

On day 15, chickens in the challenged treatments were orally gavaged with 5 mL of distilled water containing 2876 sporulated oocysts of four *Eimeria* spp., including *tenella*, *maxima*, *acervolina*, and *necatrix*. In order to intensify the induction of coccidiosis, the net floor of the cage was covered with thick plastic to prevent the waste from spilling into the trays of each floor, and the waste was remained in the bed until the end of the period to repeat the coccidiosis cycle several times. During the onset of the challenge period (third and fourth weeks), optimum house temperature and the litter moisture were controlled [25].

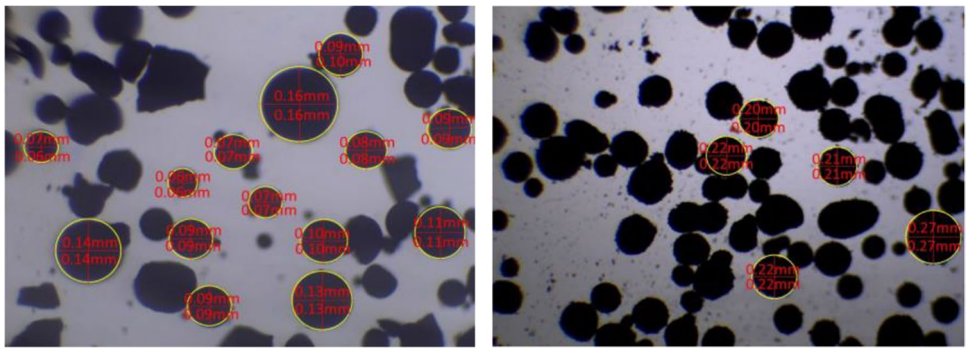

Fig. 2 Transmission electron microscopy (TEM) image of monovalent copper oxide (Cu_2O, left) and potentiated zinc oxide (ZnO, right). Measured by academy of Minatoyoor laboratory (www.minatoyoor.com). Mean particle size of Cu_2O was 0.072 mm (SD, 0.028; CV, 38.9%) and mean particle size of ZnO was 0.167 mm (SD, 0.049; CV, 29.4%). The above images show the optimal physical shape that will result in the homogeneous mixing of trace minerals with feed

Measurement of the Number of Oocysts per Gram (OPG) of Excreta

The excreta samples were collected 1 day before, and 7 days post coccidial infection from all experimental units to calculate oocyst shedding. Total excreta from each cage were mixed thoroughly to ensure uniformity. Samples were kept in the refrigerator for the determination of OPG. The oocyst counting was done followed by Mc Master Techniques as described by Chand et al. [26]. Excreta samples (2 g) were mixed in 10% (w/v) NaCl solution. The prepared suspension was poured to McMaster chamber using a micropipette and the numbers of oocysts were counted by using a microscope. No oocytes were observed in the excreta of non-challenged chickens at both stages of the measurement, which indicates the accurate isolation of experimental units and control of research operations.

Intestinal Histomorphology

The collected tissue samples were fixed in 10% neutral buffered formalin, and, following tissue processing, embedded in paraffin wax. The paraffin-embedded tissues were cut into 5-mm-thick sections using a microtome (Rotary microtome, Did Sabz company, model DS4055, Urmia, Iran) and then stained with hematoxylin and eosin (H&E). For histological observations, the height and thickness of the jejunum were assessed by optical microscopy at × 20 magnification. Setting scale and measurements were conducted using image J 1.52a software. To measure the height of the jejunum villous, cross-sections of 10 villous were randomly selected. Villous was selected based on the presence of an intact lamina propria. The length of the villous from the tip of the villous to the junction of the villous crypt and the depth of the crypt from the base of the villous to the mucosa were measured. The height (VH), width (VW), and depth (VD) of the jejunum crypt were expressed as the mean for each bird. The ratio of villous to crypts (VH:CD) was calculated by dividing the VH by the CD [27].

Intestinal Lesion Scoring

Lesion scoring of the intestine was done at the end of the trial. One bird per replicate with a similar BW to the average of the experimental unit (cage) was selected for visual lesion scoring. The lesion was evaluated based on the existence and or severity of petechial hemorrhages. The lesions were scored as recommended by Johnson and Reid [28].

Measurement of Oxidative Status Indicators

At 42 days of age, a blood sample was taken from the brachial vein of one bird from each replicate. Total antioxidant capacity (TAC) and superoxide dismutase (SOD) enzyme activity were measured by the Randox commercial kit (Randox, UK) using a Slcyon autoanalyzer [29]. Malondialdehyde (MDA) serum concentration as an antioxidant biomarker was determined by spectrophotometrically. Plasma alkaline phosphatase activity (ALP) was measured with an automatic biochemical analyzer (Hitachi 717, Boehringer Mannheim, Ingelheim am Rhein, Germany) using an Elitech Diagnostic kit (catalog no. A.110537).

Statistical Analysis

Data were analyzed using GLM procedure of SAS software (SAS 9.4 Institute Inc., Cary, NC), and means were compared by Duncan's multiple-range test. Residue normality was analyzed using the test of Shapiro Wilk (Proc. UNIVARIATE). The statistical model included Cu and Zn doses, and coccidiosis challenge as the main effects and their interaction. The following model was applied:

$$Y_{ijk} = \mu + A_i + B_j + C_k + AB_{ij} + BC_{jk} + AC_{ik} + ABC_{ijk} + e_{ijkl}$$

where μ is the general average, A_i is the Cu levels, B_j is the Zn levels, C_k is the coccidiosis challenge, AB_{ij} is the effect of the interaction between Cu and Zn levels, AC_{ik} is the effect of the interaction between Cu levels and coccidiosis challenge, BC_{jk} is the effect of the interaction between Zn levels and coccidiosis challenge interaction effect, ABC_{ijk} is the effect of the interaction among Cu and Zn levels and coccidiosis challenge, and e_{ijkl} is the incidental residual effect of observation.

The experimental unit was the cage, and statistical significance and tendencies were considered at $P \leq 0.05$ and $0.05 < P \leq 0.10$, respectively. The main effects were discussed in cases where the interaction was not significant. On the other hand, the interaction effects were discussed for responses in which the interaction was significant.

Results

Growth Performance

Average body weight (BW), feed intake (FI), and feed conversion ratio (FCR) are shown in Table 2. Supplementation of Cu_2O and ZnO had a significant effect on BW and FI. The *Eimeria* spp. challenge reduced ($P < 0.05$) BW and ADG of broilers post infection (Table 2), which confirms the completion of the oocyst cycle and the infection with coccidiosis in the challenged groups. Trace minerals did not affect FCR up to 35 days; however, added 80 mg/kg Zn improved ($P < 0.03$) FCR at day 42 (Table 2). Challenged broilers had higher ($P < 0.02$, $P < 0.04$, $P < 0.001$) FCR at days 21, 28, and 42, and tended to have higher FCR ($P < 0.06$) at day 35 than non-challenged birds. The interaction effect of supplemented trace minerals (Cu × Zn) on the FCR was significant ($P < 0.05$). The lowest and the highest values (1.53 and 1.69) were observed in chickens fed a diet containing 160 mg/kg Zn plus 150 mg/kg Cu, and 160 mg/kg Zn, respectively (interaction data not presented in the tables). The post infection mortality rate of challenged and non-challenged groups was the same (4.16%). Treatments and their interaction were not significant on mortality rate (results not presented).

Oocyst Shedding, Jejunum Histomorphology, and Liver weight

Fecal oocyst shedding was affected by the treatments and their interactions, as shown in Table 3. Added Cu_2O and ZnO linearly decreased ($P < 0.0001$) OPG in the challenged broilers (Table 3). The interaction of copper and zinc on the number of excreted oocyst was observed ($P < 0.0001$) in infected broilers (Table 6). The highest number of oocysts was excreted by the birds fed diet without added copper and zinc, and the lowest was observed in the birds fed diet contained 150 mg/kg copper from Cu_2O and 160 mg/kg zinc from potentiated ZnO (Table 6).

Infection of broilers with *Eimeria* caused a significant decrease ($P < 0.0001$) in the VH and CD of jejunum (Table 3). Added Cu_2O linearly increased ($P < 0.0001$) VH, CD, and VH:CD in broilers (Table 3). Added ZnO linearly increased ($P < 0.0001$) VH and VH:CD in broilers (Table 3). The effect of zinc supplementation on CD was significant ($P < 0.002$). The jejunum CD of broilers fed diet contained 80 mg/kg zinc was more than 0, and 160 mg/kg groups (Table 3). The interaction of copper and zinc on the VH ($P < 0.01$), CD ($P < 0.0001$), and VH:CD ($P < 0.0001$) was observed (Table 6). The VH and VH:CD were higher in the group receiving 150 mg/kg copper and 160 mg/kg zinc and the lowest value was observed in the broilers fed a diet without added trace minerals (Table 6). The crypt was deeper in the group receiving 150 mg/kg copper and 80 mg/kg zinc (Table 6).

Microscopic features of non-challenged (treatments 1–9, T1 to T9 as described in Fig. 1), and challenged (treatments 10–18, T10 to T18 as described in Fig. 1) broilers' jejunum, fed with Cu_2O and potentiated ZnO are given in Figs. 3 and 4, respectively. Non-challenged T1 jejunum showed normal length, and intact villus, and T9 (non-challenged broilers fed 150 mg/kg Cu, and 160 mg/kg Zn) showed a considerable increase in the villus length (Fig. 3). The study of jejunum histopathology for challenged chickens that were fed a diet without supplemented Cu_2O and potentiated ZnO (T10) showed looseness, sloughing, necrotic glandular, disintegrated, and shortening of villus, and shallow crypts (Fig. 4). Infected and supplemented group with Cu_2O and potentiated ZnO (treatments T11–T18) revealed improvement with increased dietary levels of copper and zinc (Fig. 4). In such a way, T12 showed relative regenerated epithelium, and T18 (challenged broilers fed 150 mg/kg copper, and 160 mg/kg zinc) showed intact and strong normal histomorphology (Fig. 4).

Different levels of copper and zinc had no significant effect on liver weight (Table 3). The fractional liver weight of *Eimeria*-infected broilers significantly ($P < 0.0001$) was higher than the healthy group (Table 3). No interaction of the main effects was observed on the liver weight at day 42.

Intestinal Lesion Score

Intestinal lesion scoring of different groups is presented in Table 4. Non-challenged groups (T1–T9) did not show any lesion, hemorrhage, and congestion. Severe intestinal hemorrhages were present in the T10, moderate hemorrhages were seen in the T11, T12, and T13, and mild hemorrhages in the T15 and T16, while there were no hemorrhages in

Table 2 Effect of treatments on growth performance (BW, FI, and FCR) of male broiler chickens[1]

Item[2]		Cu_2O, mg/kg			ZnO, mg/kg			Challenge		SEM	*P*-value						
		0	15	150	0	80	160	-	+		Cu	Zn	CH	Cu×Zn	Cu×CH	Zn×CH	Cu×Zn×CH
BW, g	d 7	132.1	135.2	135.0	135.6	130.3	136.4			2.1	0.52	0.10		0.69			
	d 14	362.1	364.4	373.3	368.4	360.1	371.4			6.4	0.45	0.45		0.95			
	d 21	874.8	841.6	861.1	866.4	832.2	878.7	869.1	849.2	18	0.49	0.23	0.38	0.88	0.78	0.66	0.98
	d 28	1339.8	1351.7	1981.3	1360.3	1341.3	1379.2	1403.4[a]	1317.2[b]	25	0.35	0.57	0.004	0.75	0.90	0.63	0.96
	d 35	1949.3	1939.5	1981.3	1950.7	1931.1	1988.2	1995.8[a]	1917.7[b]	32	0.66	0.48	0.05	0.91	0.97	0.35	0.98
	d 42	2645.2	2602.2	2670.2	2605.3	2666.5	2640.8	2731.6[a]	2546.8[b]	41	0.45	0.52	0.0001	0.52	0.39	0.82	0.89
ADG, g	d 0–42	61.98	60.96	62.58	61.03	62.49	61.88	64.04[a]	59.64[b]	4.5	0.46	0.53	0.0001	0.54	0.40	0.83	0.88
FI, g	d 0–7	95.5	96.6	96.7	96.8	93.9	98.2			2.1	0.86	0.22		0.69			
	d 0–14	398.5	399.9	406.2	403.7	399.0	401.9			7.3	0.74	0.90		0.54			
	d 0–21	979.4	965.8	989.6	980.7	960.3	993.8	973.4	983.1	19	0.72	0.52	0.68	0.81	0.84	0.51	0.85
	d 0–28	1795.7	1807.9	1798.1	1815.5	1761.3	1825.0	1826.6	1774.5	35	0.96	0.40	0.21	0.53	0.76	0.15	0.95
	d 0–35	2896.2	2912.2	2900.5	2895.3	2873.5	2940.1	2934.9	2871.0	46	0.97	0.60	0.26	0.70	0.65	0.25	0.99
	d 0–42	4242.3	4159.5	4152.6	4156.4	4117.4	4271.7	4239.1	4130.5	67	0.61	0.30	0.19	0.77	0.22	0.30	0.89
FCR, g/g	d 0–7	0.72	0.71	0.72	0.71	0.72	0.72			0.01	0.78	0.72		0.26			
	d 0–14	1.10	1.10	1.09	1.10	1.11	1.08			0.01	0.77	0.37		0.45			
	d 0–21	1.12	1.15	1.15	1.14	1.15	1.13	1.12[b]	1.16[a]	0.01	0.33	0.50	0.02	0.82	0.90	0.96	0.62
	d 0–28	1.34	1.34	1.29	1.34	1.32	1.32	1.31[b]	1.35[a]	0.01	0.20	0.78	0.04	0.59	0.42	0.29	0.51
	d 0–35	1.48	1.50	1.47	1.48	1.49	1.48	1.50[x]	1.47[y]	0.01	0.17	0.90	0.06	0.66	0.35	0.79	0.85
	d 0–42	1.61	1.60	1.56	1.60[ab]	1.55[b]	1.62[a]	1.55[b]	1.62[a]	0.01	0.14	0.03	0.001	0.05	0.69	0.41	0.23

[1]The birds were challenged with coccidiosis on day 15

[2]*BW*, body weight; *ADG*, average daily gain; *FI*, feed intake; *FCR*, feed conversion ratio; *CH*, challenge

[a,b]Means with different superscripts within a row indicate a significant difference of Cu, Zn×dose, or challenge ($P < 0.05$)

[x,y]Means with different superscripts within a row indicate a tendency toward the significance of challenge ($P < 0.1$)

Table 3 Effect of treatments on liver weight (RLW), oocyst shedding (OPG), and intestinal histomorphology (VH, CD, and VH:CD) of male broiler chickens at 42 days of age[1]

Item[2]	Cu_2O, mg/kg			ZnO, mg/kg			Challenge		SEM	P-value					
	0	15	150	0	80	160	-	+		Cu	Zn	CH	Cu×Zn	Cu×CH	Zn×CH	Cu×Zn×CH
RLW g/g	2.31	2.30	2.26	2.27	2.30	2.31	2.14^{b}	2.45^{a}	0.05	0.78	0.83	<0.0001	0.07	0.51	0.14	0.14
OPG, NO/g	59533^{a}	55667^{b}	19467^{c}	63733^{a}	37467^{b}	33467^{c}	0.00^{b}	44889^{a}	4052	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
VH, µm	1137^{c}	1273^{b}	1589^{a}	1158^{c}	1338^{b}	1503^{a}	1389^{a}	1277^{b}	0.47	0.0001	0.0001	0.0001	0.01	0.86	0.12	0.31
CD, µm	98.3^{b}	99.7^{b}	112.8^{a}	101.3^{b}	108.4^{a}	101.1^{b}	108.9^{a}	98.3^{b}	0.46	0.0001	0.002	0.0001	0.0001	0.0001	0.0001	0.23
VH:CD	12.4^{c}	13.7^{b}	15.4^{a}	12.4^{b}	13.2^{b}	15.9^{a}	13.7	13.9	0.32	0.0001	0.0001	0.52	0.0001	0.0001	0.0001	0.49

[1]In the case of OPG of Cu_2O and ZnO groups, just challenged groups were compared and values were reported

[2]*RLW*, relative liver weight; *OPG*, number of oocysts per gram of excreta; *VH*, villous height; *CD*, crypt depth; *VH:CD*, ratio of villous to crypt depth

[a–c]Means with different superscripts within a row indicate a significant difference of Cu, Zn × dose, or challenge ($P < 0.05$)

the T17 and T18. The same trend was observed in the case of intestinal congestion (Table 4).

Blood Oxidative Status Indicators

Infection of broilers with *Eimeria* caused a significant decrease ($P < 0.006$) in TAC (Table 5). Superoxide dismutase was affected ($P < 0.05$) by dietary zinc supplementation and tended to have a higher value ($P < 0.07$) in broilers fed 15 mg/kg Cu than other doses (Table 5). Supplementation of Cu_2O linearly decreased plasma ALP activity ($P < 0.007$). Treatments did not affect serum MDA concentration. No interactions between treatments on blood oxidative status indicators were observed (Table 5).

Discussion

Treatment Effects on Growth Performance

In the present experiment, the challenge with *Eimeria* oocysts established by oral gavage caused a 6.8% reduction in growth performance compared to the non-challenged broiler at day 42 (2546.8 vs 2731.6 g of BW). Furthermore, challenged broilers showed a 4.5% increase in FCR at day 42 (1.62 vs 1.55). A numerical increase of FI (2.6%) was also observed with non-challenged in comparison to challenged broilers. It has been known that coccidiosis infection in the gastrointestinal tract reduces nutrient absorption and competes for nutrients within the enterocytes. These results are consistent with the results of other researchers who observed performance impairment in broiler after a challenge by *Eimeria* spp. [30–32].

The different levels of Cu and Zn supplementation did not improve the growth performance of broilers during *Eimeria* infection. Research results indicated that growth performance can be improved by supplementing Cu and Zn in the diet. Most of the researches supplemented higher dosages of trace minerals (up to 250 mg/kg) compared to the present study [33, 34]. In the present study, due to environmental considerations, a marginal quantity of Cu and Zn [35] was used.

Several studies have reported the positive response of broilers to Cu and Zn supplementation [15, 36–38], while others reported a minimal or no effect [39, 40]. These differences can be due to several factors, such as gastrointestinal status, diet composition, and their interaction, which have an impact on response to trace mineral supplementation [40–42].

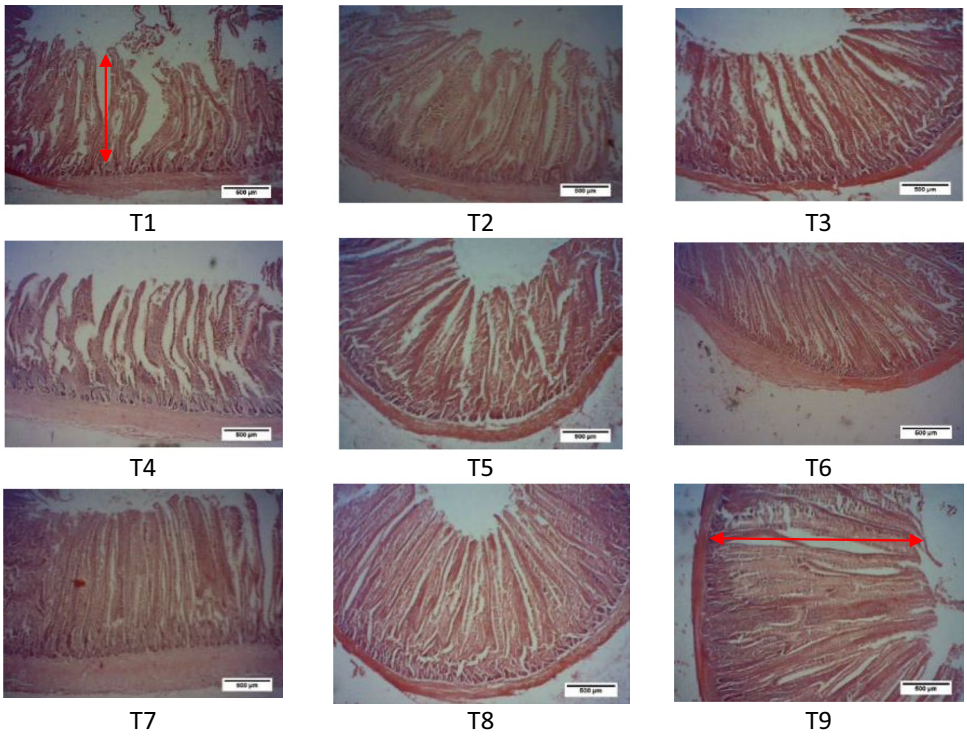

Fig. 3 Microscopic features of non-challenged broiler jejunum (treatments 1–9), microscopic image with a × 20 magnification, hematoxylin and eosin stain, scale = 500 µm. T1 jejunum showed normal length, and intact villus. T2 to T9 revealed improvement along with increased dietary levels of copper and zinc. T9 (non-challenged broilers fed 150 mg/kg Cu, and 160 mg/kg Zn) showed a considerable increase in the villus length

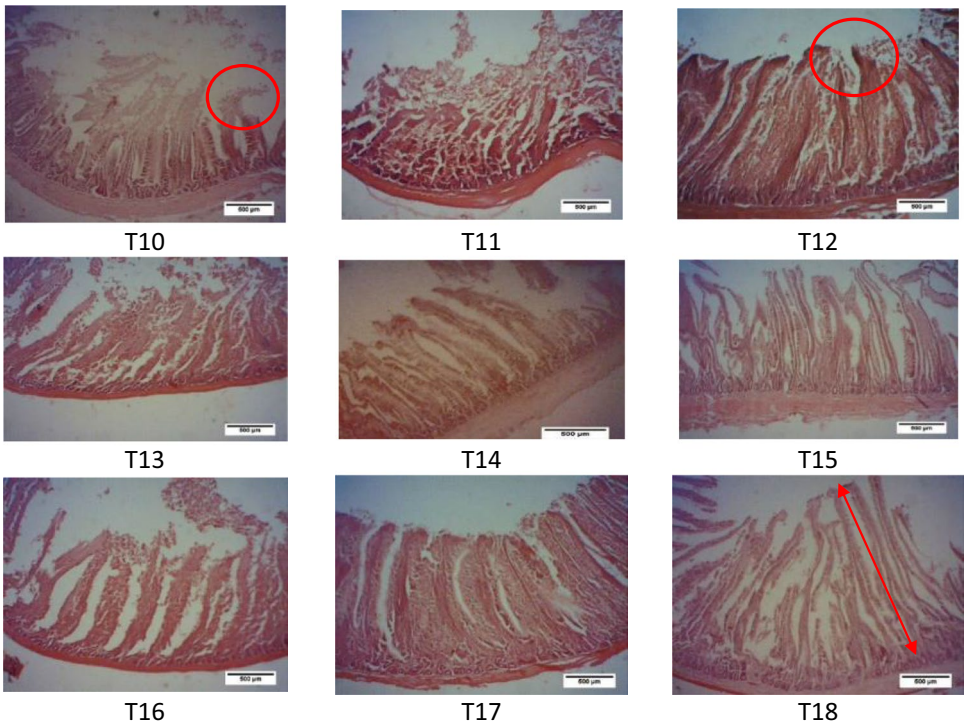

Fig. 4 Microscopic features of challenged broiler jejunum (treatments 10–18), microscopic image with a × 20 magnification, hematoxylin and eosin stain, scale = 500 µm. T10 showed looseness, sloughing, necrotic glandular, disintegrated, and shortening of villus, and shallow crypts. Treatments 11–T18 revealed improvement along with increased dietary levels of copper and zinc. T12 showed relative regenerated epithelium. T18 (challenged broilers fed 150 mg/kg copper, and 160 mg/kg zinc) showed intact and strong normal histomorphology

Copper and Zinc Effect on the Oocyst Shedding

Copper and zinc can reduce the number of oocytes excreted, but copper seems to be more effective than zinc because of its antibacterial [43] and antiviral properties [44]. The reduction on the number of excreted oocysts in broilers fed a diet supplemented with Cu_2O (150 mg/kg) and potentiated ZnO (160 mg/kg), compared to the infected group without supplementation, supports our hypothesis that Cu and Zn have a direct effect against *Eimeria*. The current

Table 4 Effect of treatments on intestinal lesion score (hemorrhages and congestion) of non-challenged (treatments 1 to 9) and challenged (treatments 10 to 18) broiler[1]

Group	Hemorrhages	Congestion
T1–T9	0	0
T10	+ + +	+ +
T11	+ +	+
T12	+ +	+
T13	+ +	+
T14	+	+
T15	+	+
T16	+	+
T17	0	0
T18	0	0

[1]Lesion scoring in the challenged groups was done according to a four-score scale, 0 = no lesion; + = mild changes; + + = moderate changes; + + + = severe changes (Johnson and Reid, 1970)

results are in agreement with the report showing that Cu and Zn supplementation minimized the shedding of *Eimeria* in infected broilers [32]. Furthermore, in a previous study, zinc nanoparticles exhibited anticoccidial activity, evidenced as a significant lowering in the output of *Eimeria papillata* oocysts within the feces of infected mice. This diminished output reflected that zinc nanoparticles impaired the development of parasites in the host before the oocysts could be formed and released [11]. Another possible mechanism is the increase in IgA levels due to Cu and Zn supplementation, which improves the intestinal defense against *Eimeria*, reducing their proliferation by binding directly to the surface of the oocysts and preventing them from attaching to the intestinal epithelium [12, 45, 46].

The synergistic effect of 150 mg/kg Cu and 160 mg/kg Zn on the shedding counts of infected broilers may be due to the facilitation of *Eimeria* membrane permeability by copper ions [47, 48], and the application of oxidant effects of zinc through the production of active oxygen species [19]. Despite copper hazard to all life [48–50], this hypothesis requires further investigations. The possible anticoccidial activity and direct damage of the *Eimeria* cells by Cu and Zn in the chickens have not been reported before.

Copper and Zinc Effect on the Gastrointestinal Histomorphology

The main objective of the current experiment was to study the influence of monovalent copper source and potentiated zinc oxide on the gut health of broiler chickens challenged with coccidiosis. The current result is in agreement with a study reporting that Cu supplementation minimized intestinal damage in broilers challenged with *Eimeria tenella* [51] (Anissimova et al., 2013). In another study

Table 5 Effect of treatments on blood oxidative status Indicators (TAC, SOD, MDA, and ALP) of male broiler chickens at 42 days of age

Item[1]	Cu_2O, mg/kg			ZnO, mg/kg			Challenge		SEM	P-value					
	0	15	150	0	80	160	-	+		Cu	Zn	CH	Cu × Zn	Cu × CH	Zn × CH	Cu × Zn × CH
TAC, mmol/L	1.36	1.36	1.39	1.38	1.35	1.38	1.28[b]	1.46[a]	0.05	0.87	0.90	0.006	0.52	0.24	0.35	0.56
SOD, U/g Hb	790.5[y]	960.8[x]	882.9[xy]	785.1[b]	965.0[a]	884.2[ab]	925.4	830.7	52	0.07	0.05	0.12	0.25	0.94	0.87	0.94
MDA, nmol/ml	2.31	2.44	2.45	2.33	2.49	2.37	2.42	2.38	0.14	0.77	0.73	0.82	0.19	0.23	0.18	0.62
ALP, U/L	1826.7[a]	1561.8[b]	1467.3[b]	1590.7	1584.0	1681.1	1648.7	1588.6	75.6	0.007	0.63	0.52	0.40	0.94	0.26	0.92

[1]*TAC*, total antioxidant capacity; *SOD*, superoxide dismutase; *MDA*, malondialdehyde; *ALP*, alkaline phosphatase

[a,b]Means with different superscripts within a row indicate a significant difference of Cu, Zn × dose, or challenge ($P < 0.05$)

[x,y]Means with different superscripts within a row indicate a tendency toward the significance of Cu × dose ($P < 0.1$)

Table 6 Interactive effect of Cu_2O, ZnO, and coccidiosis challenge on oocyst shedding (OPG), and intestinal histomorphology (VH, CD, and VH:CD) of male broiler at 42 days of age[1]

Treatment			Item[2]			
Cu_2O, mg/kg	ZnO, mg/kg	Challenge	OPG[3], NO/g	VH, µm	CD, µm	VH:CD
0	0		80400^{a}	912.5^{e}	92.2^{c}	11.02^{d}
	80		50400^{c}	1201.3^{d}	96.3^{c}	13.21^{c}
	160		47800^{d}	1298.4^{dc}	106.4^{abc}	12.98^{c}
15	0		78000^{b}	1132.9^{d}	98.7^{bc}	12.45^{cd}
	80		46800^{e}	1249.7^{d}	108.1^{abc}	12.40^{cd}
	160		42200^{f}	1436.8^{bc}	92.4^{c}	16.30^{b}
150	0		32800^{g}	1429.6^{bc}	113.1^{ab}	13.75^{c}
	80		15200^{h}	1564.6^{b}	120.7^{a}	14.00^{c}
	160		10400^{i}	1774.0^{a}	104.4^{abc}	18.46^{a}
SEM			3831	33.06	1.95	0.19
0		-		1200.1	108.7^{a}	11.55^{d}
15				1322.2	106.9^{a}	13.25^{c}
150				1646.0	111.1^{a}	16.38^{a}
0		+		1074.7	87.92^{b}	13.26^{c}
15				1224.2	92.7^{b}	14.19^{b}
150				1532.8	114.5^{a}	14.42^{b}
SEM				43.51	2.82	0.68
	0	-		1232.9	116.0^{a}	11.35^{d}
	80			1401.1	103.0^{bc}	12.99^{c}
	160			1534.3	86.7^{d}	16.84^{a}
	0	+		1083.8	96.9^{cd}	13.45^{c}
	80			1276.0	113.8^{ab}	13.43^{c}
	160			1471.9	105.3^{abc}	14.99^{b}
SEM				43.51	2.82	0.68
P-value						
Cu × Zn			< 0.0001	0.01	< 0.0001	0.0001
Cu × CH			< 0.0001	0.82	< 0.0001	< 0.0001
Zn × CH			< 0.0001	0.13	< 0.0001	< 0.0001

[1]Data of OPG are means of 4 replications of challenged groups

[2]*OPG*, number of oocysts per gram of excreta; *VH*, villous height; *CD*, crypt depth; *VH:CD*, ratio of villous to crypt depth

[3]Since the OPG value in non-challenging birds was zero, the averages interactive effects of Cu × CH and Zn × CH were not provided.[a–i]Means with different superscripts within a column indicate a significant difference ($P < 0.05$)

on broilers, Santos et al. [32] indicated that 150 mg/kg Cu supplementation significantly reduced lesion scores in the duodenum of broilers challenged with mixed *Eimeria* spp. Copper ion is toxic and can effectively kill microorganisms through an oxidation mechanism [41, 52, 53]. The electrostatic and ionic properties of Cu lead to the leakage of intracellular ions and low-molecular-weight metabolites by altering the permeability of cellular membranes [47].

The supplementation of zinc decreased the jejunum structure damages and intestinal lesion score of broilers. These observations are in line with other reports [11, 18, 19, 54] which indicated the anticoccidial and antibacterial properties of zinc oxide.

The results of the present study noted the additional benefits of using copper in combination with zinc on the improvement of gastrointestinal histomorphology of *Eimeria*-infected broilers (Table 6).

Copper and Zinc Effect on the Oxidative Status

In this experiment, infection with *Eimeria* induced a marked injury of the jejunum through the induction of oxidative stress. Copper and zinc have a role in activating the enzymes involved in the body's antioxidant defense system [35]; Cu and Zn supplementation could increase Cu and Zn availability for SOD that neutralizes free radicals

to reduce oxidative stress. Bun et al. [55] reported greater activity of SOD in broilers fed 40 and 60 mg/kg Zn at day 42, challenged with *Eimeria tenella*.

Conclusion

To conclude, Cu_2O and potentiated ZnO exhibit anticoccidial activity, due to a significant lowering in the shedding count of *Eimeria* oocysts in the excreta of infected broilers. A combination of Cu_2O (Cu 150 mg/kg) and potentiated ZnO (Zn 160 mg/kg) could be a safe option to reduce the detrimental effects of *Eimeria* infection and maintain intestinal integrity in broilers.

Author Contribution SP and MA wrote the manuscript; they took an active part in experimental research. MZ checked the manuscript and made statistical adjustments. MZH determined the elements.

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Data Availability The data of this study will be made available at reasonable request.

Declarations

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest The authors declare no competing interests.

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