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Effects of zinc dosage and particle size on gut morphology, tight junctions and TNF-α expression in broiler breeder hens

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Abstract

This study was performed to evaluate the effects of different amounts and particle size of zinc oxide (ZnO) on villus height (VH), villus width (VW), crypt depth (CD) and VH to CD ratio (VH: CD), and expression of zonula occludens-1 (ZO-1), occludin (OC) and tumour necrosis factor-α (TNF-α) in broiler breeders. A total of 350 (Ross 308) broiler breeder hens of 54 weeks randomly assigned to seven treatments, included control basal diet (C) without added Zn, C+ 100, and 130 mg Zn per kg of diet from Large (L) (100–1000 nm) and Small (S) (<100 nm) particle size ZnO (LZnO100 and 130; SZnO100 and 130), C and SZnO100 challenged with lipopolysaccharide (C+LPS and SZnO100+LPS). Each diet was fed to five replicates consisting of ten birds each. The middle part of the duodenum, jejunum and ileum was used for morphological assessments. To assess the gene expression of ZO-1, OC and TNF-α in the jejunum samples were excised. Results showed that the supplementing 130 ppm SZnO increased VH:CD in the duodenum ($p < 0.05$). VW in the duodenum and all the evaluated morphometric indices in jejunum and ileum were not affected by the dietary treatment ($p > 0.05$). ZO-1 mRNA abundance in C+LPS group compared to SZnO100+LPS group was significantly decreased and increased by LPS and SZnO100 respectively. The SZnO-100 increased OC gene expression in compare to C+LPS group. The expression of TNF-α in C+LPS treatment was higher than other groups ($p < 0.05$). The lowest and the highest litter moisture and foot-pad dermatitis (FPD) were observed in LZnO-130 and C treatments respectively ($p < 0.05$). Improving the physical properties of ZnO affect on VH:CD. Broiler breeder diet with ZnO enhance ZO-1, OC and mitigate TNF-α gene expression in jejunum maintenance of gut health in broiler breeders.

KEYWORDS
foot-pad dermatitis, gene expression, gut health, inflammation, intestinal morphology

1 | INTRODUCTION

The majority of immune cells in birds are located in the gastrointestinal tract (GIT) (Nishio, & Honda, 2012). GIT homeostasis depends on the communication among immune cells, the microbiota and the host (Buffie & Pamer, 2013; Lawley & Walker, 2013). Gut health is vital for poultry wellbeing and efficiency. In order to prevent bacterial invading to the bloodstream, the integrity of GIT is necessary to be maintained (Mullin et al., 2009). Imbalances homeostasis of GIT and gut problems can cause physiological and immunological challenges resulting in inflammatory responses (Chovatiya & Medzhitov, 2014). Daily feed restriction was used as a routine procedure to control broiler breeders body weight during rearing and laying periods. Broiler breeders are prone to gut inflammation due to fasting and emptying of GIT. The consequence of gut inflammation is the alteration in intestinal architecture, the incidence of diarrhoea, and

increased moisture in the litter (Awad et al., 2017), the most important cause of foot-pad dermatitis (FPD) (Taira et al., 2013). FPD is a form of inflammation and necrotic lesions on the plantar surface of the foot-pads with significant animal welfare, and economic implications (Shepherd & Fairchild, 2010). For years, the GIT challenges have been controlled using antibiotics (Niewold, 2007). The antibiotic growth promoting (AGP) ban on animal diets in many countries has aroused interest to study alternatives antibiotics (Gadde et al., 2017). One of the alternative compounds which have been studied is zinc oxide (ZnO) (Wang et al., 2019). Zinc (Zn) is required for intestinal function, regeneration of damaged gut epithelium (Alam et al., 1994), and improvement of intestinal injury after enteric diseases (MacDonald, 2000).

In previous studies, it was reported that Zn decreased the intestinal permeability by increasing the tight junction-related proteins expression (Li et al., 2015; Prasad et al., 2011; Troche, 2012). The consequences of Zn deficiency were inflammatory responses and production of other inflammatory cytokines (Li et al., 2015) and downregulation of the inflammatory gene expression (Hu et al., 2014).

There are different sources of Zn for animal nutrition, including $ZnSO_4$, ZnO, nano-ZnO and organic Zn. These sources have advantages and disadvantages for poultry that are mentioned. Zinc sulphate ($ZnSO_4$) is more water soluble and more available to bird, but allowing reactive metallic ions to promote free-radical formation, responsible for the breakdown of vitamins, fats and essential oils, down rating the nutritive value of the diets. (Batal et al., 2001). Zinc oxides are less reactive, but are again less bioavailable for poultry (Batal et al., 2001). The surface area of Nano-ZnO is higher than the conventional ZnO (Padmavathy & Vijayaraghavan, 2008) which can positively affect growth performance in livestock and poultry (Mishra et al., 2014), however, the widespread use of it can have short-term and long-term effects on human health (Czyżowska & Barbasz, 2020). Organic Zn (organic acids Zn and amino acid complexes Zn) has a higher bioavailability and leads better health and performance in poultry, however, the price of these sources is higher than inorganic sources (Huang et al., 2009). It is reported that one of the most effective alternatives to antibiotics is a high level of dietary conventional ZnO in pig's diet (Sales, 2013), however, it increases the excretion of Zn into the environment and causes potential environmental pollution (Broom et al., 2006) and development of Zn resistance in the gut bacteria (Cavaco et al., 2011). According to these cases, it is necessary to consider a source of ZnO with unique physical properties that can provide equal efficacy at lower dosages compared to conventional ZnO. In an ex vivo study, Vahjen et al. (2012) reported that the solubility and bacterial growth reduction were higher in chyme from donor piglets that were supplemented with unique ZnO sources (with different physicochemical features, such as high specific surface area, unique particle size and shape, large agglomerates and small aggregates), compared to analytical ZnO at the same concentration. Since no research has been done on broiler breeder hens in this field and given that the most production and reproduction problems occur from week 50 onwards, the aim of the study was to evaluate the effect of ZnO dosage and particle size (large and small sizes) on gut morphology, tight junction proteins and TNF-α gene expression in broiler breeder hens both in basal condition and after a LPS challenge.

2 | MATERIALS AND METHODS

2.1 | Birds and treatments

A total of 350 Ross broiler breeder hens were selected at 54 weeks of age from a commercial flock. The hens were randomly distributed into seven treatments include basal Control diet (C) without supplemental Zn, C+ 100 and 130 mg Zn per kg of diet from the Large (L) particle size of ZnO (LZnO100 and 130), C+ 100, and 130 mg Zn per kg of diet from the Small (S) particle size of ZnO (SZnO100 and 130), C and SZnO100 challenged with lipopolysaccharide (C+LPS and SZnO100+LPS). Hens in LPS group were received LPS orally from *Escherichia coli* O111:B4 (L2630, Sigma- Aldrich) at a dose of 250 mg/kg body weight according to Wu et al. (2013) and 96 hours before slaughter.

Composition of the basal diet was shown in Table 1. Each diet was fed to five replicates consisting of ten birds each. In this study, we used a conventional ZnO with large particle size (LZnO) and a unique ZnO with small particle size (SZnO). The purity of SZnO and LZnO was 76% and their characteristics are compared in Table 2 and Figure 1. Transmission electron microscopy (TEM) image of two sources of zinc oxide are given in Figure 1. 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 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 air at room temperature. Trial started at 54 and terminated at 65 weeks of age (12 weeks experiment). Each replicate was housed in floor pens furnished with wood shaving, a manual round feeder and one bell drinker. Water and feed were offered *ad libitum* and restricted respectively. Mash diets formulated for hens following the Ross 308 recommendation (Aviagen Group Ltd., 2016). The basal diet contained 26.5 mg Zn/kg, which was determined by the atomic absorption spectroscopy. All birds are kept in a controlled environment house under a lighting programme of 13.5 h of continuous light. An ambient temperature of 22–23°C was maintained by controlled ventilation and heating. All birds fed a depletion diet (without added Zn) for two weeks prior to start of the dietary treatments.

2.2 | Sample collection and morphology evaluation

At the end of the experimental period, two hens per experimental unit were selected randomly and slaughtered for necropsy. For measurements of the small intestine morphological characteristics, the middle part of the duodenum, jejunum and ileum were taken

TABLE 1 Ingredients and chemical composition of the basal diets fed to breeder hens (As fed basis)

Ingredients	Amount (g/kg)
Corn	723.6
Soybean meal (CP = 44%)	169.8
Corn oil	3.1
Dicalcium phosphate	12.6
Mineral oyster shall	79.8
Common salt	3.4
$NaHCO_3$	1.0
Mineral premixes[1]	2.5
Vitamin premix[2]	2.5
DL-Methionine, 99%	1.4
L-Threonine	0.2
Calculated nutrient content	
ME (Mj/kg)	11.72
CP (g/kg)	130
Calcium (g/kg)	34
Available phosphorus (g/kg)	3.2
Sodium (g/kg)	1.8
(Na+K)-Cl (meq/kg)	162
Dig. Methionine (g/kg)[3]	3.5
Dig. Methionine+Cysteine (g/kg)	5.4
Dig. Lysine (g/kg)	5.2
Dig. Threonine (g/kg)	4.7
Zn (mg/Kg)[4]	26.5

[1]Provides (per kg of diet): Copper ($CuSO_4 \cdot 5H_2O$), 10 mg; iodine (CaI), 2 mg; iron ($FeSO_4 \cdot 4H_2O$), 50 mg; manganese ($MnSO_4 \cdot H_2O$), 120 mg; selenium (Na_2SeO_3), 0.3 mg, Zn (ZnO), 0 mg.

[2]Provides (per kg of diet): Retinyl acetate (vitamin A), 11000 IU; cholecalciferol (vitamin D_3), 3500 IU; DL-α-tocopheryl acetate (vitamin E), 100 IU; menadione (vitamin K_3), 5.0 mg; thiamine (vitamin B_1), 3.0 mg; riboflavin (vitamin B_2), 12 mg; D-pantothenic acid (vitamin B5), 15 mg; niacin (vitamin B_3), 55 mg; pyridoxine (vitamin B_6), 4 mg; biotin (vitamin B_7), 0.25 mg; folic acid (vitamin B_9), 2 mg; cobalamin (vitamin B_{12}), 0.03 mg, Choline (as choline chloride): 300 mg.

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

[4]Zinc was analysed by atomic absorption spectrophotometry.

and rinsed in physiological serum. All the samples were fixed in 10% buffered formalin and stored until morphological evaluation. Each tissue was placed into a tissue cassette and was processed using dehydration protocol through a series of graded alcohols, which were then cleared with xylene and embedded in paraffin. Afterwards, 5 μm thick cross-sections (longitudinal sections) of samples were mounted onto slides. Slides were then stained by Hematoxylin and Eosin (Luna, 1968). Ten villi were measured for each intestinal segment for each bird by using a light microscope at ×10 magnification and DinoCapture 2.0 camera. The following morphometric indices were measured: villus height (VH) (villus tip to crypt opening), villus width (VW) and crypt depth (CD) (crypt opening to the base of crypt

TABLE 2 Comparison of LZnO and SZnO physical characteristics (Noori et al., 2019)

Characteristics	LZnO[1]	SZnO[2]
Particle size (nm)	100–1000	<100
Area to weight ratio (m^2/g)	2.4	42
CV (%)[3]	5.61	3.65
Angle of repose (degree)[4]	35	28
Mixability	Poor	Good

[1]Large particle size of ZnO.

[2]Small particle size of ZnO.

[3]Coefficient of variation of Zn in complete feed.

[4]The angle of repose or critical angle of repose of a granular material is the steepest angle of descent or dip relative to the horizontal plane to which a material can be piled without slumping. (25-30 excellent, 31-35 good flowability) (Comply Scientific, 2016).

right before the lamina propria) using ImageJ software (Version 2.1). Villus height to crypt depth ratios (VH: CD) was then calculated.

2.3 | RNA extraction, *cDNA* synthesis and gene expression (Real-time quantitative PCR)

ZO-1, OC and TNF-α gene expression was assessed in jejunum samples. Jejunal tissue was homogenized with pestle and mortar under liquid nitrogen and total RNA extracted using RNeasy Mini Kit (Dena Zist Asia Co.,) according to the manufacturer's instructions. RNA quantity was determined by spectrophotometry (NanoDrop-1000, Thermo Fisher Scientific). Prior to complementary DNA (cDNA) synthesis, DNAse treatment was applied. RNA (2–5 μl) was treated with 1.5 μl of DNase and 1.5 μl of buffer for 30 min. at 37°C. The DNAse was inactivated by the addition of 1.5 μl EDTA at 65°C for 5–10 min. Two micrograms of total RNA were used to synthesize the first-strand cDNA using the High Capacity cDNA Reverse Transcription Kit (Pars Tous Co.,) according to the manufacturer's recommendation. Suitable primers were designed using the GenBank sequences deposited on the NCBI and US National Library of Medicine shown in Table 3.

The PCR amplifications were done in a final volume of 15 μl reaction mixture containing 1 μl of cDNA, 7.5 μl RealQ plus 2x master mix green (Ampliqon), 0.6 μl (10 μmol/L) of each primer, and 5.3 μl sterilized water, using Rotor-Gene Q System (QIAGEN Hilden). Gene expression was analysed using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housing keeping gene. Average gene expression relative to GAPDH for each sample was calculated using the $2^{-\Delta\Delta Ct}$ method. The calibrator for each gene was the average ΔCt value from the negative control group (Pender et al., 2017).

2.4 | Litter moisture and foot-pad dermatitis measurement

In order to assess the gut integrity, the litter moisture and foot-pad dermatitis were measured. Five samples of litter were collected from

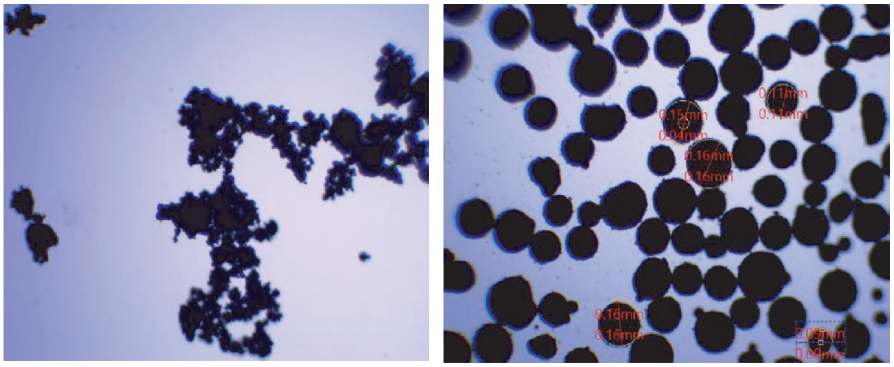

FIGURE 1 Transmission electron microscopy (TEM) image of two sources of zinc oxide (Right, SZnO and Left, LZnO). (Measured by academy of Minatoyoor laboratory, www.minatoyoor.com)

TABLE 3 Gene-specific primers for real-time quantitative reverse transcription PCR

Gene name	Primer sequence	Product size (bp)	Annealing temperature (°C)	GenBank Accession
ZO-1[1]	F: GCCCTTAAAGAGGAAGCTGTG	208	60	HAEK01045424
	R: GTGAAGAGTCACCGTGTGTTG			
OC[2]	F: TCGCCTCCATCGTCTACATC	323	58	D21837
	R: GTTCTTCACCCACTCCTCCA			
TNF-α[3]	F: CTGTTTCTGCCTCTGCCATC	192	60	AY765397
	R: GGGTTCATTCCCTTCCCATCT			
GAPDH[4]	F: CCATCACAGCCACACAGAAG	201	58	AF047874
	R: AGGTCAGGTCAACAACAGAGA			

[1]Zonula Occluden-1.
[2]Occludin.
[3]Tumour Necrosis Factor-α.
[4]Glyceraldehyde 3-Phosphate Dehydrogenase.

each pen and then mixed to obtain a single pool for the analysis of litter moisture content. Foot-pad dermatitis evaluation was carried out by the method described by Michel et al. (2012), in this method FDP was divided into three types; type I: mild lesions (visually determined by enlargement and erythema of the crust); type II: moderate ulcers (visually determined by a hypertrophic and hyperkeratotic lesion covered with yellowish to brownish exudations); type III: conspicuous lesions (visually determined by a thick dark adherent crust and broad ulceration).

2.5 | Statistical analysis

The data were analysed by Proc GLM of SAS 9.1. (SAS Institute, 2011) software with pen means used as an experimental unit. Residue normality was analysed using the test of Shapiro–Wilk (Proc UNIVARIATE). Results are presented as the Lsmean ±SE. The Turkey's test was applied to compare Lsmeans the level of significance adjusted to $p < 0.05$. The one-way model is:

$Y_{ij} = \mu + T_i + e_{ij}$.

Where:

Y_{ij}: observation j in treatment i.

μ: the overall mean.

T_i: the fixed effect of treatment i.

e_{ij}: random error with mean 0 and variance δ^2.

3 | RESULTS

3.1 | Small intestine morphology

The impact of different amounts and particle size of ZnO on VH, VW, CD and VH: CD in different sections of intestine at week 65 are presented in Table 4. VW in the duodenum and all the evaluated morphometric indices in jejunum and ileum were not affected by the dietary treatment ($p > 0.05$). Based on data presented in Table 4, the highest VH, CD and VH: CD in the duodenum were shown in SZnO130, LZnO100 and SZnO130 respectively. The lowest VH in the duodenum was observed in the C+LPS and SZnO100+LPS.

3.2 | Gene expression of ZO-1, OC and TNF-α in the jejunum

The effects of amounts and particle sizes of ZnO on ZO-1, OC and TNF-α gene expression in jejunum are presented in Figures 2–4. There were a significant differences in tight junction proteins and pro-inflammatory cytokine gene expression among the treatments ($p < 0.05$). The highest and the lowest ZO-1 expression was observed in SPZnO130 and C+LPS respectively (Figure 2). Results indicate that 100 ppm SZnO, increased ZO-1 mRNA abundance in

TABLE 4 The effects of different levels and particle size of ZnO on villus height (VH), villus width (VW), crypt depth (CD) and villus height to crypt depth ratio (VH:CD) in duodenum, jejunum and ileum at week 65 (μm)[1]

	Duodenum				Jejunum				Ileum			
Treatments	VH	VW	CD	VH:CD	VH	VW	CD	VH:CD	VH	VW	CD	VH:CD
C[2]	526.15^{ab}	51.43	75.02^{abc}	7.033^{b}	473.47	61.14	48.44	9.764	336.69	44.96	44.29	7.655
C+LPS[3]	388.60^{c}	47.51	64.93^{bc}	6.024^{b}	360.23	51.95	44.16	8.261	289.79	54.67	41.40	7.005
LZnO100[4]	458.34^{bc}	45.82	79.24^{a}	5.808^{b}	437.93	53.12	49.77	8.800	319.03	51.31	39.40	8.098
SZnO100[5]	459.10^{bc}	45.55	77.69^{ab}	5.909^{b}	385.78	49.59	58.13	7.045	292.28	49.34	44.66	6.721
SZnO100+LPS[6]	373.88^{c}	39.22	65.24^{bc}	5.779^{b}	347.11	53.73	47.96	7.245	251.39	55.08	38.38	6.615
LZnO130[7]	456.00^{bc}	34.07	70.54^{abc}	6.469^{b}	424.71	53.65	54.68	7.777	331.71	51.19	42.30	7.907
SZnO130[8]	551.25^{a}	52.72	63.99^{c}	8.640^{a}	478.99	56.18	53.34	9.164	301.16	47.77	45.04	6.813
SEM[9]	18.02	4.61	2.91	0.334	34.00	3.38	4.24	0.788	22.94	3.33	3.32	0.508
p-Value	0.000	0.144	0.001	0.000	0.057	0.325	0.332	0.148	0.206	0.427	0.658	0.383

[1], [a–d]Means in a column with different superscripts differ significantly ($p < 0.05$).

[2]Basal Diet (Control (C), 0 mg zinc per kg of diet).

[3]Basal Diet (Control (C), 0 mg zinc per kg of diet), challenged with Lipopolysaccharide (LPS).

[4]C+100 mg zinc per kg of diet from Large particle size of Zinc oxide.

[5]C+100 mg zinc per kg of diet from Small particle size of Zinc oxide.

[6]C+100 mg zinc per kg of diet from Small particle size of Zinc oxide, challenged with LPS.

[7]C+130 mg zinc per kg of diet from Large particle size of Zinc oxide.

[8]C+130 mg zinc per kg of diet from Small particle size of Zinc oxide.

[9]Standard error of means.

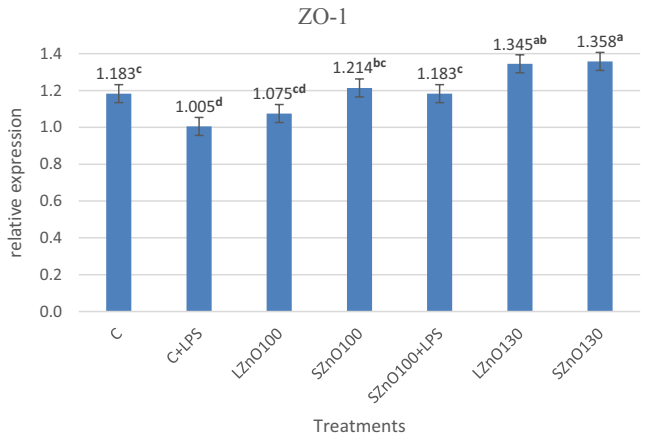

FIGURE 2 The effects of amounts and particle sizes of ZnO on ZO-1 expression in broiler breeder's jejunum[1] (P: 0.0001, SEM[2]: 0.033). [1a–d] Means in a column with different superscripts differ significantly ($p < 0.05$), C: Basal Diet (Control (C), 0 mg zinc per kg of diet), C+LPS: Basal Diet (Control (C), 0 mg zinc per kg of diet), challenged with Lipopolysaccharide (LPS), LZnO100: C+100 mg zinc per kg of diet from Large particle size of Zinc oxide, SZnO100: C+100 mg zinc per kg of diet from Small particle size of Zinc oxide, SZnO100+LPS: C+100 mg zinc per kg of diet from Small particle size of Zinc oxide, challenged with LPS, LZnO130: C+130 mg zinc per kg of diet from Large particle size of Zinc oxide, SZnO130: C+130 mg zinc per kg of diet from Small particle size of Zinc oxide, [2]Standard error of means

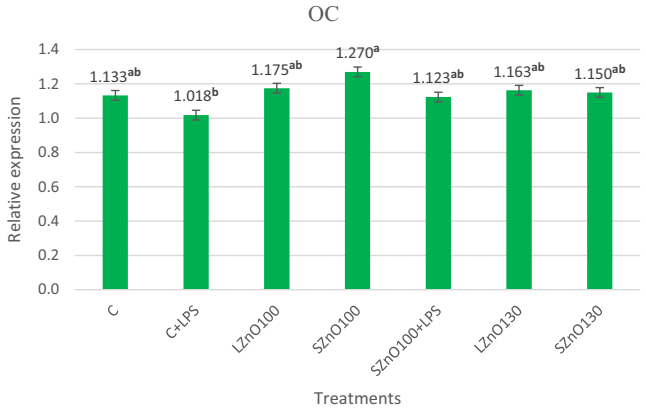

FIGURE 3 The effects of amounts and particle sizes of ZnO on OC expression in broiler breeder's jejunum[1] (P: 0.0069, SEM[2]: 0.038). [1a, b] Means in a column with different superscripts differ significantly ($p < 0.05$), C: Basal Diet (Control (C), 0 mg zinc per kg of diet), C+LPS: Basal Diet (Control (C), 0 mg zinc per kg of diet), challenged with Lipopolysaccharide (LPS), LZnO100: C+100 mg zinc per kg of diet from Large particle size of Zinc oxide, SZnO100: C+100 mg zinc per kg of diet from Small particle size of Zinc oxide, SZnO100+LPS: C+100 mg zinc per kg of diet from Small particle size of Zinc oxide, challenged with LPS, LZnO130: C+130 mg zinc per kg of diet from Large particle size of Zinc oxide, SZnO130: C+130 mg zinc per kg of diet from Small particle size of Zinc oxide, [2]Standard error of means

hens challenged with LPS (SZnO100+LPS) compare to C+LPS significantly ($p < 0.05$). The SZnO-100 increased OC gene expression in compare to C+LPS group (Figure 3). The administration of Zn in hens challenged with LPS (SZnO100+LPS) increased OC gene expression compared to C+LPS. As it is shown in Figure 4, the lowest TNF-α gene expression was observed in the C group and the highest in the C+LPS ($p < 0.05$).

3.3 | Litter moisture and foot-pad dermatitis score

The effects of different amounts and particle sizes of ZnO on litter moisture percentage and FPD score are presented in Table 5. Adding breeder hens diet with ZnO decreased litter moisture and improved foot-pad score accordingly ($p < 0.05$). In such a way the best result was observed with LZnO130. Obtained results showed that (Table 5), the severity of FPD was higher in C+LPS which was ameliorated by 100 ppm of Zn in SZnO100+LPS group.

4 | DISCUSSION

4.1 | Small Intestine morphology

Daily feed restriction was used to control broiler breeders body weight but this method prone hens to gut inflammation due to fasting. On the contrary, anti-nutritional factor existing in the feed including beta-conglycinin, present in soybean meal, as well as cereal

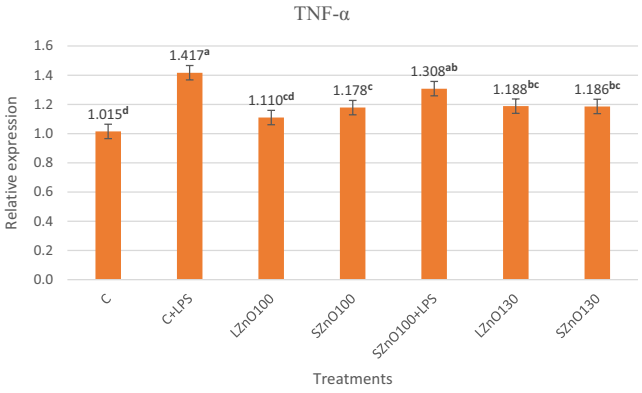

FIGURE 4 The effects of amounts and particle sizes of ZnO on TNF-α expression in broiler breeder's jejunum[1] (P: 0.0001, SEM[2]: 0.028). [1a-d] Means in a column with different superscripts differ significantly ($p < 0.05$).C: Basal Diet (Control (C), 0 mg zinc per kg of diet), C+LPS: Basal Diet (Control (C), 0 mg zinc per kg of diet), challenged with Lipopolysaccharide (LPS), LZnO100: C+100 mg zinc per kg of diet from Large particle size of Zinc oxide, SZnO100: C+100 mg zinc per kg of diet from Small particle size of Zinc oxide, SZnO100+LPS: C+100 mg zinc per kg of diet from Small particle size of Zinc oxide, challenged with LPS, LZnO130: C+130 mg zinc per kg of diet from Large particle size of Zinc oxide, SZnO130: C+130 mg zinc per kg of diet from Small particle size of Zinc oxide, [2]Standard error of means

TABLE 5 The effects of amounts and particle size of ZnO on litter moisture (%) and foot-pad dermatitis score in broiler breeders[1]

Treatments	Litter moisture (%)	Foot-pad dermatitis
C[2]	76.72[a]	1.933[ab]
C+LPS[3]	65.91[a]	2.100[a]
LZnO100[4]	61.92[abc]	1.560[bc]
SZnO100[5]	63.50[ab]	1.432[bc]
SZnO100+LPS[6]	76.41[a]	1.367[c]
LZnO130[7]	48.03[c]	1.200[c]
SZnO130[8]	51.25[bc]	1.440[bc]
SEM[9]	4.69	0.164
p-Value	0.001	0.021

[1], [a–d]Means in a column with different superscripts differ significantly ($p < 0.05$).

[2]Basal Diet (Control (C), 0 mg zinc per kg of diet).

[3]Basal Diet (Control (C), 0 mg zinc per kg of diet), challenged with Lipopolysaccharide (LPS).

[4]C+100 mg zinc per kg of diet from Large particle size of Zinc oxide.

[5]C+100 mg zinc per kg of diet from Small particle size of Zinc oxide.

[6]C+100 mg zinc per kg of diet from Small particle size of Zinc oxide, challenged with LPS.

[7]C+130 mg zinc per kg of diet from Large particle size of Zinc oxide.

[8]C+130 mg zinc per kg of diet from Small particle size of Zinc oxide.

[9]Standard error of means.

gluten prolamins play a role of inflammatory agents. This is a low-grade chronic inflammation due to non-infectious stimuli can lead to gut inflammation (Peng et al., 2020). Therefore, broiler breeders are exposed to stressors under normal conditions. However, to better compare the effect of stressors on hens intestinal, LPS was used in some treatments to induce extreme inflammation. It is reported that Zn is essential for normal intestinal barrier function and the regeneration of damaged gut epithelium (Alam et al., 1994). In the current experiment, the results showed that supplementation of hens diet with Zn had positive effects on intestinal architecture. In particular supplementing 130 ppm SZnO increased VH:CD in the duodenum. These results were consistent with Shah et al. (2019) who reported that by supplementation of Zn and probiotic in broiler diet the villi height in the duodenum was increased. This is associated with the increased proliferation of crypt cells due to Zn availability.

In our previous study, it was reported that SZnO100 and 130 increase egg production rate, settable egg production and eggshell thickness in comparison with LZnO100 and 130 (Barzegar et al., 2020). As mentioned before, in comparison with LZnO, SZnO has different physicochemical properties, such as high specific surface area, unique particle size and shape, large agglomerates, and small aggregates. It is mentioned that the different physicochemical characteristics of ZnO sources can affect their solubility and cause variable bioavailability (Wang et al., 2019). In the present study improved in the main intestinal morphological criteria (VH:CD) by adding SZnO or LZnO were supported by the following findings. Feng et al. (2010) showed that Zn is necessary for protein synthesis, development of intestinal cells, improvement of absorptive capacity, and growth performance enhancement. With supplementation of 80 and 120 mg Zn/kg of diet the VH:CD in broilers challenged with *Salmonella typhimurium* was higher (Zhang et al., 2012), due to rapid turnover of epithelial cell. VH and VH:CD are appropriate indicators of intestinal morphology (Lei et al., 2014). More mitotic activity in the villi increases its length and absorptive surface (Onderci et al., 2006; Samanya & Yamauchi, 2002). Rapid metabolism of villi tissue increases crypt depth that indicating the replacement of new cells and their regeneration (Hamedi et al., 2011). Higher VH:CD refers to an increase in nutrient digestibility and improve absorption capacity in chickens (Silva et al., 2009). On the other hand, SZnO has antibacterial properties against microorganisms, including Gram-positive and Gram-negative bacteria (Ahmadi et al., 2020). The most common antibacterial action of SZnO is the generation of ions from the surface of SZnO and bind to electron donor groups on the bacterial cell surface and damage the cell membrane (Aviagen Group Ltd., 2020) and the other is generation of reactive oxygen species (ROS), which induces oxidative stress and cell death (Aviagen Group Ltd., 2020). It seems that the supplementation of SZnO in broiler breeder diet improved intestinal morphology by effect on pathogenic microorganism.

In this study, villus height decreased in the duodenum of LPS-challenged birds. LPS is a large molecule consisting of a lipid and a polysaccharide found in the outer membrane of Gram-negative bacteria that can cause damage to intestinal tissue, immune response and physiological changes. From an intestinal physiological point of view, LPS decreased the VH and VH: CD in the duodenum, increased the CD of the ileum (Li et al., 2015) and decreased jejunal VH in early LPS-challenged chicks (Zhang et al.,2012), However, Li et al. (2015) did not observe that LPS affected the jejunum, which is not consistent with our study. The VH and VH: CD ratio were increased and the CD was decreased in the small intestine of weaned piglets with a dietary high level of ZnO (Li et al., 2001), due to an increase in protein synthesis and cell proliferation in the intestine (Neto et al., 2011). In agreement with these findings, our study demonstrated that Zn reduced the damages to breeders gut tissue and repairs the intestine mucosal. This indicates an increase in the absorptive capacity of the small intestine, which is necessary for optimal production in broiler breeders.

5 | GENE EXPRESSION OF ZO-1, OC AND TNF-α IN THE JEJUNUM

Since the gut barrier integrity is necessary for the normal functions of the gut to protect the poultry from pathogens we used ZnO to evaluate potential effects of ZnO on gut health. Previous studies have shown that the ZnO and infeed antibiotics are effective on development of small intestine, intestinal immune-associated gene expression, regulate the antioxidant capacity and growth performance in weaned piglets (Hill et al., 2001; Zhu et al., 2017). In the present study improved in the tight junction proteins gene expression (ZO-1

and OC increase) and mitigation of breeder gut inflammation (TNF-α decrease) by adding SZnO or LZnO. Wen et al. (2018) found that the transcription of the intestinal barrier-related genes was upregulated with supplementation of Zn to duck diets. Our results and others were consistent with the previous studies that zinc played a vital role in maintaining epithelial cell integrity in weaned piglets (Zhu et al., 2017), rats (Sturniolo et al.,2002) and children (Roy et al., 1992). The use of high doses of ZnO supplementation upregulated the mRNA expression of ZO-1 and OC in the jejunum (Zhu et al., 2017) and ileum (Zhang & Guo, 2009) mucosa of weaned piglets. Increased tight junction expression indicates a repair mechanism in monolayer epithelial cells (Akbari et al., 2014). Other impacts of Zn on cell membrane integrity probably involve the regulation of redox status (Srivastava et al., 1995) by preventing apoptosis in the small intestine (Wang et al., 2009). However, this parameter was not examined in the present study. Multiple environmental factors (intestinal pathogens, poor quality feed ingredients, changes in feed formulation etc.) of commercial production can trigger gut inflammation (Kogut et al., 2018). Inflammation is caused by innate immune system when detects and identifies the infection, dangerous molecules and host damage (Chovatiya & Medzhitov, 2014), resulted in production of a number of cells and pro-inflammatory molecules (such as TNF-α) (Barton, 2008). LPS is recognized by toll-like receptor-4 (TLR-4) (Mani et al., 2012) and activates the nuclear factor kappa B (NF-κB), a protein that controls gene transcription of pro-inflammatory cytokines including TNF-α, IL-1 and IL-6, promote an inflammatory response (Tan et al., 2014). It is reported that ZnO upregulate mRNA expression of inflammatory cytokines (e.g. IL-8 and TNF-α) induced by enterotoxigenic E. coli (ETEC) K88 in human colon Caco-2 cells (Roselli et al., 2003), in porcine intestinal epithelial IPEC-J2 cells (Sargeant et al., 2011) and in weaned mouse intestine (Ren et al., 2014). Therefore, although stress could upregulate the expression of pro-inflammatory cytokines, the use of anti-inflammatory compounds such as Zn can prevent the negative effects of inflammation. A previous study showed that high dietary Zn downregulated the TLR4 expression and the pro-inflammatory cytokine IL-8 in the colon of weaned piglets (Liu et al., 2014). An earlier study reported that Zn protects the cells against TNF-α-induced disruption of the monolayer cell (Hennig et al., 1993). Darsi et al. (2021) reported that the supply of 70, 100 mgZn/Kg of diet by SZnO and LZnO significantly increased and decreased the TNF-α in broiler breeders serum respectively. Thus the amount of inflammatory factors in hens that are prone to intestinal inflammation due to the once a day feeding decreases with the consumption of higher levels of Zn. In mammals, Zn deficiency causes to a decrease in A20 abundance and subsequently impairs the gut mucosa barrier (Morgan et al., 2011). Zinc finger protein A20 by deubiquitinating ubiquitin-dependent factors of NFκB signalling can negatively effects on inflammatory response (Catrysse et al., 2014). It is reported that supplementation of Zn in broiler breeders diet can improved intestinal morphological characteristics in progeny by DNA hypomethylation and histone H3 at lysine 9 (H3K9) hyperacetylation at the A20 promoter region, thus adding of Zn in maternal dietary exhibited greater attenuation of gut impairment and decreased the abundance of TNF-α and activate A20 (Li et al., 2015).

5.1 | Litter moisture and foot-pad dermatitis

There are many factors that effects on occurrence of FPD (De Jong et al., 2012; Meluzzi et al., 2008; Skrbic et al., 2015); however, poor quality litter and nutrition of birds are the most important causes of FPD (Da Costa et al., 2014; Taira et al., 2013). According to our results 130 ppm Zn from LZnO led to decreased litter moisture and FPD. It is demonstrated that biotin and Zn, as cofactors of essential enzymes for protein and nucleic acid synthesis, are important for the improvement of the skin status. Abd El-Wahab et al. (2013) reported that a diet containing, 150 mg Zn from organic sources and 2000 g biotin per kg of diet can reduce the severity of FPD. Similarly, Youssef et al. (2012) found that supplementation of high biotin or Zn in the diet of female turkeys can alleviate the severity of FPD. In the current study, litter moisture and FPD have been increased in birds challenged with LPS in comparison with other treatments. According to intestine morphology results, a challenge with LPS led to intestinal problems and diarrhoea, which results in increased litter moisture that is reduced by the use of Zn. It means that effects of LPS on intestine were attenuated by increment levels of Zn. On the other hand, Zn plays an important role in the formation of collagen in the skin, which leads to maintaining tight junctions among skin cells (Saenmahayak et al., 2010).

In our knowledge, there were no data or any research projects on broiler breeder gut inflammation that was one of the main limitation of our literature review. Therefore, obtained results of present study compare with data on other animals.

6 | CONCLUSIONS

In conclusion, our results indicated that improving the physical properties of ZnO (SZnO) affected in VH:CD. Broiler breeders diet with ZnO enhance ZO-1 and OC and mitigate TNF-α gene expression in jejunum that led to improvement of intestinal barrier integrity and maintenance of gut health, which resulted in a decrease in litter moisture and the prevalence of FPD.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

ANIMAL WELFARE STATEMENT

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. The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, upon reasonable request, subject to restrictions and conditions.

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