

Effects of dietary supplementation of lipid-coated zinc oxide on intestinal mucosal morphology and expression of the genes associated with growth and immune function in weanling pigs

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Objective: The present study was conducted to investigate the effects of a lipid-coated zinc oxide (ZnO) supplement Shield Zn (SZ) at the sub-pharmacological concentration on intestinal morphology and gene expression in weanling pigs, with an aim to gain insights into the mechanism of actions for SZ.

Methods: Forty 22-day-old weanling pigs were fed a nursery diet supplemented with 100 or 2,500 mg Zn/kg with uncoated ZnO (negative control [NC] or positive control [PC], respectively), 100, 200, or 400 mg Zn/kg with SZ for 14 days and their intestinal tissues were taken for histological and molecular biological examinations. The villus height (VH) and crypt depth (CD) of the intestinal mucosa were measured microscopically following preparation of the tissue specimen; expression of the genes associated with growth and immune function was determined using the real-time quantitative polymerase chain reaction.

Results: There was no difference in daily gain, gain:feed, and diarrhea score between the SZ group and either of NC and PC. The VH and VH:CD ratio were less for the SZ group vs NC in the jejunum and duodenum, respectively ($p < 0.05$). The jejunal mucosal mRNA levels of insulin-like growth factor (IGF-I) and interleukin (IL)-10 regressed and tended to regress ($p = 0.053$) on the SZ concentration with a positive coefficient, respectively, whereas the IL-6 mRNA level regressed on the SZ concentration with a negative coefficient. The mRNA levels of IGF-I, zonula occludens protein-1, tumor necrosis factor- α , IL-6, and IL-10 did not differ between the SZ group and either of NC and PC; the occludin and transforming growth factor- β 1 mRNA levels were lower for the SZ group than for PC.

Conclusion: The present results are interpreted to suggest that dietary ZnO provided by SZ may play a role in intestinal mucosal growth and immune function by modulating the expression of *IGF-I*, *IL-6*, and *IL-10* genes.

Keywords: Post-weaning Pig; Zinc Oxide; Intestine; Morphology; Gene Expression; Immunity

INTRODUCTION

Zinc oxide (ZnO) is added to the nursery pig diet at 2,000 to 4,000 mg/kg to alleviate the diarrhea and growth check of the post-weaning piglets in many non-EU countries [1-3]. However, such a pharmacological ZnO supplementation poses a hazard of environmental pollution with the heavy metal, because dietary ZnO is mostly excreted in feces due to low absorption efficiency [4,5]. It is therefore necessary to find any Zn supplement that can elicit the effects of pharmacological ZnO at a lower dose. Shield Zn (SZ) used in the feeding trial of the present study is a proprietary ZnO product in which the mineral particle is coated with lipid to maximize the rate of delivery of the mineral in its native chemical form to the

intestine without being ionized in the stomach.

The present ZnO study group has reported that dietary supplementation of 100 mg Zn/kg with SZ ('basal SZ') had no beneficial effect on fecal consistency (diarrhea) score (FCS) and intestinal villus structure, with no or slightly growth-enhancing effect, compared with those with basal ZnO in weanling pigs whereas pharmacological ZnO (2,500 mg Zn/kg) increased the daily gain and reduced FCS without affecting the villus structure [6,7]. Moreover, the circulating and hepatic Zn concentrations of the piglets were greater in the pharmacological ZnO group than in the basal SZ and basal ZnO-100 groups, with no difference between the latter two groups, in these studies as well as in a latest study with post-weaning pigs challenged with enterotoxigenic *Escherichia coli* (ETEC) K88 (Han JH, unpublished results). The growth-enhancing effect of basal SZ observed in weanling piglets [7], as well as the growth-enhancing and diarrhea-alleviating effects of the supplementation in the ETEC K88-challenged piglets [8,9], are therefore likely to have resulted from the action of SZ in the intestine before its complete absorption into general circulation. Little is known, however, about the molecular mechanism of actions of SZ at the intestine as well as the dose effect of the Zn supplement. The present study was therefore performed to investigate the effects of SZ supplemented at the sub-pharmacological concentration compared with those with basal as well as pharmacological ZnO and also the dose effects of SZ on the intestinal mucosal morphology and expression of the genes associated with growth and immunity in weanling pigs and, thereby, to gain insights into the mechanism of actions of the Zn supplement.

MATERIALS AND METHODS

Animals and dietary treatments

The protocol for the present experiment was approved by the Institutional Animal Care and Use Committee of Gyeongnam National University of Science and Technology. Forty castrated piglets born to Duroc-sired Yorkshire×Landrace dams were allotted randomly to five dietary treatments in 40 pens, with eight pens per treatment, immediately after weaning at 21 days of age. The dietary treatments were supplementations to a basal nursery diet (Table 1) with 100 mg Zn/kg as ZnO (basal ZnO or negative control [NC]), 2,500 mg Zn/kg as ZnO (pharmacological ZnO or positive control [PC]), 100, 200, or 400 mg Zn with Shield Zn (SZ-100, -200, or -400, respectively; CTCBIO, Seoul, Korea). The basal diet was formulated to meet or exceed the nutrient requirements for 5 to 10 kg piglets recommended by the Committee on Korean Feeding Standard for Swine [10]. The piglets were adapted to the experimental diet overnight and placed on a 14-day feeding trial on the following day. The diet and water were freely accessible to the piglets throughout the experiment. The ambient temperature

Table 1. Composition of the basal diet (as-fed basis)

Component	Content
Ingredient (%)	
Corn	33.16
Barley	8.0
Soybean meal	10.0
Dehulled soybean meal	10.0
Fermented soybean	4.15
Sweet whey	12.56
Lactose	4.20
Fish meal	5.00
Wheat bran	3.00
Sugar	3.00
Soy oil	3.00
Organic acids	0.70
Limestone	0.30
Monocalcium phosphate	1.20
Salt	0.30
Vitamin premix ¹⁾	0.15
Mineral premix ²⁾ (Zn-free)	0.20
Others ³⁾	0.77
Total ⁴⁾	99.69
Calculated chemical composition	
Digestible energy (Mcal/kg)	3.50
Crude protein (%)	18.50
Ether extract (%)	4.50
Lysine (%)	1.52

¹⁾ Provided per kg: 1,500 IU vitamin A; 2,000 IU vitamin D₃; 65 IU vitamin E; 1.5 mg vitamin K; 1.0 mg thiamin; 6 mg riboflavin; 20 mg pantothenic acid; 25 mg niacin; 1.5 mg vitamin B₆; 1 mg folic acid; 25 µg vitamin B¹²; 25 µg biotin; and 150 mg choline.

²⁾ Provided per kg: 160 mg Cu; 200 mg Fe; 40 mg Mn; 1.0 mg I; 0.15 mg Co; and 0.4 mg Se.

³⁾ Provided per total weight: 0.10% choline-HCl; 0.35% L-lysine-HCl (78%); 0.15% DL-methionine (99%); 0.11% L-threonine (99%); 0.01% L-tryptophan; and 0.05% ethoxyquin.

⁴⁾ Five experimental diets were supplemented to the basal diet with 125 mg ZnO; 3,125 mg ZnO; 139 mg of 10% lipid (w/w)-coated ZnO (Shield Zn [SZ], CTCBIO, Seoul); 278 mg SZ; and 556 mg SZ per kg diet, respectively, as well as 0 to 0.30% corn, to provide 100, 2,500, 100, 200, and 400 mg Zn/kg, respectively.

was controlled at 30°C and 29°C during the first and second weeks of the trial, respectively. The body weight and feed intake were measured on days 0, 7, and 14 and on days 7 and 14, respectively. The fecal consistency was subjectively scored as previously described [6,7] except that a 0-to-2 scale was used in the present study instead of the 1-to-3 scale: 0, normal firm feces; 1, soft feces; 2, diarrhea.

Histological examination

All piglets were euthanized by electric stunning at the end of the feeding trial and the intestinal tissue was taken from each of the segments of the duodenum, jejunum, ileum, and colon also as described previously [8,11,12]. The tissue was fixed in a 10% neutral formalin solution, embedded, mounted onto the slide, and stained, after which the villus height (VH) and

crypt depth (CD) of each of the small intestinal segments, as well as the goblet cell density of the colon, were measured under the microscopic field.

Real-time quantitative polymerase chain reaction

Total RNA was extracted from the jejunal mucosa using RNeasy Lysis Buffer (TRIzol Reagent, Invitrogen, Eugene, OR, USA). The levels of the target gene mRNA and the internal control β -actin gene were determined by real-time quantitative polymerase chain reaction (PCR) with SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) after the synthesis of the first-strand DNA using the cDNA Cycle kit (Invitrogen Co., Carlsbad, CA, USA) as previously described [13,14]. The nucleotide sequences of the forward and reverse primers for zonula occludens protein-1 (ZO-1) were 5'-CCTGAGTTTGATAGTGGCGTTGA-3' and 5'-AAATAGATTTTCCTGCCCAATTCC-3', respectively; those for interleukin (IL)-10 were 5'-GCATCCACTTCCCAA CCA-3' and 5'-CTTCCTCATCTTCATCGTCAT-3', respectively. For the primers for insulin-like growth factor-I (IGF-I), occludin, IL-6, tumor necrosis factor- α (TNF- α), and transforming growth factor- β 1 (TGF- β 1), the nucleotide sequences designed by Shen et al [15] were used. The PCR conditions were initial denaturation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The relative level of the target gene expression was normalized to that for the control gene according to the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen [16].

Statistical analysis

The effects of the total SZ treatment (SZ-100, -200, and -400) compared with those of NC or PC were analyzed via contrast using the general linear model procedure of SAS (SAS Institute Inc., Cary, NC, USA). The piglet was the experimental unit in all variables. For the FCS, in which the model included the day and day \times treatment interaction in addition to the treatment, the effects of the day and the day \times treatment interaction were tested using day \times piglet as the error term. The linear and quadratic regressions of each variable on the dietary SZ concentration within the total SZ treatment were analyzed using the regression procedure of SAS. The significance and tendency of the results of the regression and contrast were defined as $p < 0.05$ and $0.05 \leq p < 0.10$, respectively.

RESULTS

Growth performance and FCS

The average daily gain (ADG) did not differ between the SZ group and either of the NC and PC groups during the period of days 0 to 7, 7 to 14, or 0 to 14 (Table 2). The average daily feed intake, which did not differ between the SZ and NC groups for any of the experimental periods, tended to be less for the

SZ group vs PC during the period of days 7 to 14 and was also less for the former group for the entire 14-day period. On the other hand, the gain:feed ratio tended to be greater for the SZ group vs PC during the period of days 7 to 14.

The majority of the animals exhibited a low FCS corresponding to the normal firm feces throughout the experiment. The FCS did not differ between the SZ group and either of the NC and PC groups on any of days 0, 7, and 14 or for the entire experimental period. Nevertheless, the mean FCS increased between days 0 and 7 and returned to the day-0 level by day 14 (0.10, 0.30, and 0.15 for days 0, 7, and 14, respectively, with standard error of mean = 0.065).

Intestinal mucosal morphology

The VH in the duodenum tended to be less for the SZ group vs NC whereas the CD was greater for the former (Table 3). The VH:CD ratio in the duodenum was less for the SZ group than for both NC and PC groups. In the jejunum, the VH was less for the SZ group vs NC group, but the CD and VH:CD ratio did not differ between the SZ group vs either of the NC and PC groups. In the ileum, neither the VH nor CD differed between the SZ and either of the NC and PC groups, but the VH:CD ratio tended to be less for the SZ group vs ZnO-100. The goblet cell density in the colon did not differ between the SZ group and either of the NC and PC groups.

Gene expression

The IGF-I mRNA level in the jejunal mucosa did not differ between the SZ group and either of the NC and PC groups (Table 4). Within the SZ group, the IGF-I mRNA level regressed on the supplemental SZ concentration with a positive coefficient. The mRNA level of ZO-1 did not differ between the SZ group and either of the NC and PC groups. The occludin mRNA level for the SZ group did not differ from the level for the NC group, but it was lower than that for the PC group. The mRNA levels of TNF- α , IL-6, and IL-10 did not differ between the SZ group and either of the NC and PC groups. Within the SZ group, the IL-6 mRNA level regressed on the supplemental SZ concentration with a negative coefficient whereas the IL-10 mRNA level tended to regress on the supplemental SZ concentration with a positive coefficient ($p = 0.053$). The TGF- β 1 mRNA level did not differ between the SZ and NC groups, but it was lower for the SZ group vs PC.

DISCUSSION

The lack of any significant effect of the SZ treatment as well as PC vs NC on growth performance and FCS was consistent with the results observed by Jang et al [6] in which the piglets were housed in small 4-animal pens at the same farm where the present feeding trial was performed. However, these results differed from the increased ADG and decreased FCS in

Table 2. Comparative effects of lipid-coated ZnO (Shield Zn [SZ]) vs uncoated ZnO on growth performance and fecal consistency of weaning pigs

Variable	ZnO ¹⁾		SZ ¹⁾			SEM	p-value			
	100 (NC)	2,500 (PC)	100	200	400		Contrast		Regression ³⁾	
							NC ²⁾	PC ²⁾	L	Q
BW (kg)										
Day 0	5.13	5.81	5.74	5.36	5.50	0.21	0.010	0.262	0.524	0.651
Day 7	6.22	7.26	6.97	6.89	6.47	0.30	0.113	0.171	0.162	0.154
Day 14	8.51	9.49	9.21	9.39	8.67	0.35	0.156	0.325	0.119	0.089
ADG (g)										
Days 0-7	156	207	175	219	138	25	0.456	0.315	0.138	0.079
Days 7-14	326	319	319	356	315	20	0.869	0.647	0.600	0.442
Overall	241	263	247	288	226	17	0.529	0.651	0.147	0.070
ADFI (g)										
Days 0-7	245	288	258	265	237	19	0.706	0.122	0.312	0.273
Days 7-14	599	689	592	652	574	35	0.867	0.051	0.470	0.340
Overall	422	488	425	458	405	25	0.791	0.048	0.356	0.259
Gain:feed										
Days 0-7	0.620	0.711	0.653	0.821	0.608	0.077	0.414	0.852	0.442	0.301
Days 7-14	0.546	0.473	0.547	0.559	0.559	0.040	0.849	0.081	0.862	0.882
Overall	0.570	0.546	0.585	0.635	0.570	0.039	0.556	0.267	0.610	0.501
FCS ⁴⁾										
Day 0	0.13	0.25	0.00	0.00	0.13	-	-	-	-	-
Day 7	0.25	0.50	0.13	0.38	0.25	0.15 ⁵⁾	-	-	-	-
Day 14	0.25	0.13	0.13	0.13	0.13	-	-	-	-	-
Overall ⁶⁾	0.21	0.29	0.08	0.17	0.17	0.09	0.478	0.121	0.473	0.535

NC, negative control; PC, positive control; SEM, standard error of mean; L, linear; Q, quadratic; BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; FCS, fecal consistency score.

¹⁾ The numeral under the column heading indicates the dietary Zn concentration in mg/kg provided by the Zn supplement. Data are the means of 8 piglets.

²⁾ Total SZ (SZ-100, -200, and -400) vs NC and total SZ vs PC, respectively.

³⁾ Linear and quadratic regressions on the dietary SZ concentration within the total SZ group piglets were analyzed separately.

⁴⁾ Scored subjectively: 0, formal firm feces; 1, soft feces; 2, diarrhea.

⁵⁾ Applies to all day × treatment combinations.

⁶⁾ The p values for the day and day × treatment were 0.086 and 0.915, respectively.

response to basal SZ in the piglets challenged with ETEC K88 [8,9] as well as the increased ADG due to the treatment in unchallenged piglets in large pens (34 piglets/pen) under commercial setting [7]. It thus seems apparent from these results that the effects of the sub-pharmacological dose of SZ as well as pharmacological ZnO on growth performance and FCS in post-weaning pigs vary, depending on both the status of infection with ETEC and the pen size.

The VH/VH:CD ratio and CD are commonly used as positive and negative indices, respectively, for the structural integrity of the intestinal mucosa [3,5,17]. As such, the smaller or tendency of smaller VH or VH:CD ratio for the SZ group vs NC is suggestive of a negative effect of dietary SZ on the integrity of the villus structure. However, this may as well be taken as a tentative suggestion, because the VH or VH:CD ratio occasionally decreased in response to a physiological dose of lipid-coated ZnO [6] or pharmacological ZnO ([18]; present study [the smaller ileal VH:CD ratio for the PC vs NC group]), which commonly exhibited a positive or no effect on these structural variables [8,9,19-21]. Accordingly, for the present result

of the SZ effect to be substantiated, it needs to be confirmed unequivocally in future studies.

Intestinal mucosal growth and immunity are known to be regulated by a number of growth factors [22,23], structural proteins [24], and cytokines [25-27]. Of those growth factors and structural proteins, IGF-I and the tight junction proteins ZO-1 and occludin play central roles in mucosal growth and the barrier function against the microbial dissemination, respectively. It is also well known that TNF- α and IL-6 stimulate inflammatory responses whereas the anti-inflammatory cytokines TGF- β 1 and IL-10 suppress them [28,29]. The lower mRNA levels of occludin and TGF- β 1 for the SZ group vs PC observed in the present study then suggest that SZ-100 to -400 is less potent than pharmacological ZnO in inducing the expression of the structural protein and cytokine. Moreover, the regression and the tendency of regression of the IGF-I and IL-10 mRNA levels on the supplemental SZ concentration, respectively, suggest that the expression of these peptides may be up-regulated by SZ. Likewise, IL-6 expression is seemingly down-regulated by the increasing dose of SZ, as suggested by

Table 3. Comparative effects of lipid-coated ZnO (Shield Zn [SZ]) vs uncoated ZnO on morphology of the gastrointestinal tract of weanling pigs

Variable	ZnO ¹⁾		SZ ¹⁾			SEM	p-value			
	100 (NC)	2,500 (PC)	100	200	400		Contrast		Regression ³⁾	
							NC ²⁾	PC ²⁾	L	Q
Duodenum										
VH (µm)	302	280	280	272	278	11	0.056	0.813	0.948	0.983
CD (µm)	246	253	276	290	273	14	0.046	0.111	0.701	0.609
VH:CD	1.22	1.15	1.02	0.96	1.02	0.06	0.002	0.027	0.827	0.713
Jejunum										
VH (µm)	261	238	228	240	232	12	0.048	0.710	0.923	0.985
CD (µm)	206	191	184	199	191	11	0.218	0.986	0.777	0.889
VH:CD	1.27	1.28	1.25	1.22	1.22	0.07	0.558	0.501	0.960	0.791
Ileum										
VH	222	202	213	240	209	12	0.951	0.178	0.593	0.434
CD	163	170	175	190	167	11	0.282	0.597	0.411	0.312
VH:CD	1.39	1.20	1.21	1.29	1.26	0.06	0.074	0.451	0.744	0.833
Colon (cell density [cells/mm ²])										
Goblet cell	956	980	920	962	819	69	0.490	0.325	0.314	0.271

NC, negative control; PC, positive control; SEM, standard error of mean; L, linear; Q, quadratic; VH, villus height; CD, crypt depth.

¹⁾ The numeral under the column heading indicates the dietary Zn concentration in mg/kg provided by the Zn supplement. Data are the means of 8 piglets.

²⁾ Total SZ (SZ-100, -200, and -400) vs NC and total SZ vs. PC, respectively.

³⁾ Linear and quadratic regressions on the dietary SZ concentration within the total SZ group piglets were analyzed separately.

the negative regression of its mRNA level on the supplemental SZ concentration. Shen et al [15] and Grilli et al [30] have reported the effects for one of two other lipid-coated ZnO supplements, respectively, on jejunal expression of a number of cytokines [30] as well as growth factors and tight junction proteins [15], but comparison of their results with the present ones was precluded because of the unknown efficacies of the different Zn supplements as well as different ranges of dietary concentrations of them examined in these and present studies.

Only limited information is available as to the effects of the lipid-coated ZnO supplement on expression of the genes in the intestine in association with its main function. Pharmacological ZnO, however, is known to increase the intestinal

expression of the genes of IGF-I, tight junction proteins, and anti-inflammatory cytokines [31-35], which has been interpreted to suggest that the ZnO supplementation improves the mucosal morphology and barrier function and also modulates the immune function. Shen et al [25] reported similar effects for a lipid-coated ZnO supplement, but such effects were apparent only within a certain range of supra-physiological concentrations. The present results also are thought to be similar to the suggested actions for pharmacological ZnO; however, this was suggested by the regression or trend of the mRNA levels of the genes on the dietary SZ concentration rather than by the changes of the mRNA levels in response to SZ vs basal ZnO, except for the results for occludin which

Table 4. Comparative effects of lipid-coated ZnO (Shield Zn [SZ]) vs uncoated ZnO on gene expressions in the jejunal mucosa of weanling pigs

Variable	ZnO ¹⁾		SZ ¹⁾			SEM	p-value			
	100 (NC)	2,500 (PC)	100	200	400		Contrast		Regression ³⁾	
							NC ²⁾	PC ²⁾	L	Q
<i>IGF-I</i>	1.00	2.09	0.58	1.27	2.21	0.54	0.572	0.248	0.003	0.004
<i>ZO-1</i>	1.00	3.59	1.16	2.03	3.01	1.39	0.511	0.351	0.142	0.157
<i>Occludin</i>	1.00	1.99	0.95	0.53	0.72	0.39	0.562	0.009	0.605	0.743
<i>TNF-α</i>	1.00	1.70	0.39	1.17	1.33	0.56	0.951	0.261	0.163	0.210
<i>IL-6</i>	1.00	1.28	2.31	0.99	0.65	0.38	0.477	0.929	0.017	0.033
<i>IL-10</i>	1.00	2.87	0.37	1.12	1.92	0.99	0.906	0.141	0.053	0.062
<i>TGF-β1</i>	1.00	2.52	0.87	1.07	1.47	0.36	0.740	0.002	0.119	0.123

NC, negative control; PC, positive control; SEM, standard error of mean; L, linear; Q, quadratic; IGF-I, insulin-like growth factor-I; ZO-1, zonula occludens protein-1; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; TGF-β1, transforming growth factor-β1.

¹⁾ The numeral under the column heading indicates the dietary Zn concentration in mg/kg provided by the Zn supplement. Data are the means of 8 piglets.

²⁾ Total SZ (SZ-100, -200, and -400) vs. NC and total SZ vs PC, respectively.

³⁾ Linear and quadratic regressions on the dietary SZ concentration within the total SZ group piglets were analyzed separately.

exhibited no trend of the dose-responsiveness. Obviously, however, more studies are necessary to work out the dose effects of SZ on intestinal expression of the genes as related to growth and mucosal barrier function and immunity.

Collectively, the results of the present study are suggestive of the following conclusions. Dietary supplementation with neither the sub-pharmacological dose of SZ nor pharmacological ZnO has any significant effect on growth performance or FCS for weanling pigs when the piglets are housed individually. Dietary SZ may cause a decrease in VH or VH:CD ratio of the small intestine, which needs to be confirmed in future studies. Finally, dietary SZ may play a role in growth and inflammatory responses of the intestinal mucosa by modulating the expression of *IGF-I*, *IL-6*, and *IL-10* genes in post-weaning pigs.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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