The Effects of Dietary Porous Zinc Oxide Supplementation on Growth Performance, Inflammatory Cytokines and Tight Junction’s Gene Expression in Early-Weaned Piglets

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Summary This study was conducted to investigate the effect of dietary porous ZnO supplementation on the growth performance, inflammatory cytokines and tight junction’s gene expression in weaned piglets. A total of 192 weaned piglets were randomly allocated to 4 experimental groups (n=48/group) and fed, during 14 d, with one of the following dietary treatments: 1) basal diet (NC); 2) basal diet with 3,000 mg/kg of conventional ZnO (PC); 3) basal diet with 750 mg/kg of porous ZnO (low inclusion porous ZnO, LP-ZnO); 4) basal diet with 1,500 mg/kg porous ZnO (high inclusion porous ZnO, HP-ZnO). Results showed that dietary supplementation with regular ZnO or porous ZnO (750 and 1,500 mg/kg) improved average daily gain (ADG), feed to gain ratio (F/G) and jejunum morphology, while decreasing diarrhea incidence. Compared with the NC group, porous ZnO at both doses (750 or 1,500 mg/kg) increased serum alkaline phosphatase (ALP), immunoglobulin G (IgG) and insulin-like growth factor 1 (IGF-1) concentrations, but decreased serum glucose (GLU). Moreover, the mRNA expression of anti-inflammation cytokine (TGF-b), tight junction (Occludin, ZO-1) in the jejunum by different ZnO administration were significantly increased compared with the NC group, while mRNA expression of pro-inflammatory (IL-8), membrane channels that transport water (AQP3) and miR-122a were significantly decreased. It can be concluded that porous ZnO even at low dose (750 mg/kg) can be an effective alternative to pharmacological (3,000 mg/kg) conventional ZnO in reducing diarrhea, promoting the growth performance, increasing anti-inflammatory cytokines and tight junctions, reducing pro-inflammatory cytokines of weaned piglets.

Key Words porous ZnO, weaned piglets, barrier function, immune function, diarrhea

Post-weaning diarrhea (PWD), a major healthcare concern in swine production, results in growth retardation of piglets as consequence of the disturbance of the gastrointestinal tract function (1, 2). In-feed zinc oxide has been used as alleviator for PWD in piglets for many years (3–5), but its application has been overcommitted and caused environmental pollution (6). It is evident that zinc, as a component of many digestive enzymes and metalloenzymes, plays a key role in growth, nutrient’s metabolism and immunity and is an essential trace element for animals (7). Dietary pharmacological supplementation of zinc oxide (ZnO) can improve growth performance and inhibit intestinal inflammation in weaned piglets (8). In the last decades, various Zn forms (i.e., organic, coated ZnO, nano ZnO) provide an effective alternative for dietary pharmacological concentrations of ZnO in weaned piglets (9–13). Moreover, dietary ZnO supplementation recommended by the National Research Council (NRC 1998) for diarrhea prophylaxis was 3,000 mg/kg during the first 2 wk after weaning. However, in 2012, the NRC review the dosage of ZnO to 1,500–3,000 mg/kg and that may also be lower in the future. In addition, antibiotics have been banned in many countries as disease-fighting growth promoters in animal production. Thus, looking for effective alternatives to in-feed ZnO becomes important and urgent.

Recently, due to its increased surface area, uniform particle size, high mixing uniformity, decreased cross contamination and enhanced reactivity, porous ZnO
possess numerous advantages and can be considered as one of the most highly recommended substitutes for the conventional ZnO (14). Porous ZnO is widely applied in many areas, such as bio-medical field, plastics, and feed additive (14). Evidence has shown that the efficacy of porous ZnO in increasing the growth performance and reducing fecal scores (10, 15). However, mechanisms for the growth-promoting and anti-diarrhea impacts of porous ZnO remained not fully clear. Therefore, the study was conducted to compare porous ZnO at different dosages (750 or 1,500 mg/kg) with pharmacological level of conventional ZnO, in starter diets on growth performance, inflammatory cytokines and tight junction’s gene expression in weaned pigs.

**METHODS**

**Ethical statement.** All procedures involving animal subjects were approved by the Animal Care and Use Committee of Hunan Agricultural University (approval number 20180914, Changsha, China).

**Animals and experimental design.** One hundred and ninety-two healthy pigs weaned at 21 d (6.30±0.11 kg BW) (Duroc×Landrace×Large White) (Tangrenshen Group Co. Ltd., Zhuzhou, Hunan, China) were randomly allocated into four groups, each group with 6 replicates and 8 piglets per replicate (male and female half). The four experimental treatment groups were as follows: basal diet (Treatment 1, NC); basal diet with 3,000 mg/kg conventional zinc oxide (Hubei Bohua Agriculture and Animal Husbandry Science and Technology Co., Ltd., Hubei, China; zinc content ≥79%) (Treatment 2, PC); basal diet with 750 mg/kg porous zinc oxide (Amine SAS, Annecy, France; zinc content ≥75%) (Treatment 3, LP-ZnO); basal diet with 1,500 mg/kg porous zinc oxide (Treatment 4, HP-ZnO). Diets in this study were formulated according to the National Research Council (NRC 2012) (Table 1). The Zn content (measured value) in each diet was 139 mg/kg, 2,509 mg/kg, 807 mg/kg, 1,296 mg/kg, respectively. Throughout the experiment, piglets were given free access to feed and drinking water. The trial lasted 2 wk and feed intake, BW and diarrhea rate were measured. Average daily feed intake (ADFI), average daily gain (ADG) and feed to gain ratio (F/G) were calculated.

**Sample preparation.** On day 15, six middle-weighted piglets from each treatment (3 male and 3 females, 9.64±0.11 kg BW) were chosen to take blood from the jugular vein and sacrificed using an electric stunner, after a 12 h fasting period, according to the method of Lee et al. (16). Middle jejunum samples were collected immediately frozen in liquid nitrogen and then stored at −80°C for subsequent analyses. One piece of jejunum sample was fixed in 10% neutral buffered formalin for testing jejunal morphology.

**Intestinal histomorphology.** Villus height (VH) and crypt depth (CD) of jejunal samples were estimated via a light microscope according to a previous study of Kwon et al. (17). Briefly, the fixed intestinal segment was embedded in paraffin, after which the tissue specimen was sliced to a thickness of 4 μm, mounted onto a glass slide for hematoxylin/eosin staining. The average of four measurements was considered as a replicate for VH and CD variables.

**Blood physiological and biochemical parameters.** Serum total protein (TP), total cholesterol (TC), alkaline phosphatase (ALP), glucose (GLU), alanine aminotransferase (ALT), aspartate aminotransferase (AST), immunoglobulin M (IgM), and immunoglobulin G (IgG) in the samples were tested by spectrophotometric kits (Alchemy Biotech Development Co., Ltd., Hunan, China). Growth hormone (GH), and insulin-like growth factor-1 (IGF-1) in the serum samples were tested by ELISA kits (Wuhan Huamei Biotech Co., Ltd., Hubei, China).

**Real-time PCR.** Total RNA extraction from jejunal samples were tested by ELISA kits (Alchemy Biotech Development Co., Ltd., Hunan, China). Growth hormone (GH), and insulin-like growth factor-1 (IGF-1) in the serum samples were tested by ELISA kits (Wuhan Huamei Biotech Co., Ltd., Hubei, China).

**RESULTS**

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samples and the reverse transcription were conducted according to previous reports (18–20). Inflammatory cytokines [tumor necrosis factor α (TNF-α), transforming growth factor β (TGF-β), interleukins 8 (IL-8)], tight junctions (Occludin, ZO-1), membrane channels that transport water (AQP3) and miR-122a genes expression were determined. Primers used in this study were designed according to the gene sequence of pigs (Table 2). β-actin was used as a housekeeping gene to normalize the target gene expression of TNF-α, TGF-β, IL-8, Occludin, ZO-1, AQP3, and U6 was used as a housekeeping gene to normalize the target gene expression of miR-122a. The formula \(2^{-\Delta\Delta Ct} \), where \(\Delta\Delta Ct = (Ct_{\text{Target}} - Ct_{\beta-\text{actin}})\) treatment – \((Ct_{\text{Target}} - Ct_{\beta-\text{actin}})\) control was used to calculated the relative gene expression (21–23).

**Statistical analysis.** One-way ANOVA was used to analyze all data (SAS 8.0). In case of significant difference, a Tukey test was conducted to differentiate the mean values of the groups. Results were expressed as the mean±SD. \(p\) values <0.05 means statistical significance in this study.

**RESULTS**

**Growth performance**

Growth performance of piglets is summarized in Table 3. There was no significant difference in initial BW and final BW among the four treatment groups. However, compared with the NC treatment group, the final BW in the three Zn supplemented groups increased (\(p=0.079\))

| Table 2. Primers used for quantitative reverse transcription-PCR. |
|---|
| Primer name | Primer sequence | Size (bp) | Gene ID |
| β-Actin | F: GTTCGAGACCTCAACACCC | 181 | 414396 |
| | R: CCGCGAGGCAGGTTCCAGA | 86 | 397236 |
| Occludin | F: GGCAGGCCACCAAGACA | 148 | 396567 |
| | R: CTTTCCGCTGTCAGGATGTTT | 161 | 397086 |
| ZO-1 | F: AGCCCGAGGGTGTTTAA | 148 | 397078 |
| | R: AGGTGGGAGGATGCTGTTGT | 179 | 396880 |
| TNF-α | F: GCTGCCTGTCAGGATGTTT | 162 | 10012635 |
| TGF-β | F: CAGCTCCGATTTAACCCCTAGCC | 179 | 10012635 |
| IL-8 | F: TCCACACCTTACACCC | 224 | EU520423 |
| AQP3 | F: CATCTTTGCAATACCCCCG | 224 | EU520423 |
| U6 | F: CACTATTGCGGGTCTGC | 224 | EU520423 |
| miR-122a | F: GCCTGGAGTGTGACAATGGTG | 100 | 170404 |

1 bp: base pair.

| Table 3. Effects of porous zinc oxide on the growth performance of early-weaned piglets. |
|---|
| Items | NC | PC | LP-ZnO | HP-ZnO | \(p\)-value |
| Initial body weight (kg) | 6.37±0.95 | 6.29±0.81 | 6.22±0.82 | 6.40±0.80 | 0.985 |
| Final body weight (kg) | 9.16±0.56 | 9.93±0.40 | 9.50±0.32 | 9.97±0.36 | 0.079 |
| Average daily gain (g/d) | 199.6±30.3b | 256.5±16.6a | 234.2±8.8a | 254.9±20.6a | 0.001 |
| Average daily feed intake (g/d) | 286.5±24.4 | 314.0±32.2 | 311.5±13.8 | 319.9±21.3 | 0.121 |
| Feed to gain ratio | 1.45±0.14a | 1.21±0.08b | 1.33±0.10ab | 1.26±0.08b | 0.003 |

1 Data shown as the mean±SD, \(n=6\).

**Fig. 1.** Effects of porous zinc oxide supplementation on diarrhea incidence in the weaned piglets. Data shown as the mean±SD, \(n=6\). \(a,b\) Mean values bearing different superscript letters were significantly different (\(p<0.05\)).
by 8.4%, 3.8% and 8.8%, respectively. Compared to Control subjects, piglets fed with ZnO had significantly greater ADG (p<0.05). Although significant difference in ADFI was failed to notice among the experimental groups, feed intake increased by 9.6%, 8.73% and 11.66% in PC, LP-ZnO, and HP-ZnO groups comparing with the NC group, respectively. The F/G significantly (p<0.005) differed for PC and HP-ZnO and the NC group.

The diarrhea incidence of piglets is presented in Fig. 1. In particular, compared with the NC group, the diarrhea rate in piglets fed ZnO was remarkably decreased (p<0.05) by 57.12%, 32.10% and 44.81% in PC, LP-ZnO and HP-ZnO, respectively. Moreover, no significant differences in diarrhea rate among PC, LP-ZnO and HP-ZnO groups were observed in this study.

**Intestinal morphology**

As shown in Table 4, it was evident that an improvement of jejunal morphologic structure has happen in the piglets supplemented with 3,000 mg/kg conventional ZnO and 750 or 1,500 mg/kg of porous ZnO. Furthermore, compared with the NC treatment, villus height in PC and LP-ZnO groups was remarkably increased (p<0.05). However, we failed to notice any significant differences about the jejunal crypt depth and the ratio of jejunal villus height to crypt depth among the four treatments.

**Serum biochemical, physiological parameters and Zn concentration**

Data on serum biochemical and immune parameters are summarized in Table 5 and Fig. 2. The concentrations of serum AST, ALP, GLU, and IgG were markedly changed in function of the dietary treatments in this study.

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### Table 4. Effects of porous zinc oxide on jejunal mucosal morphology of early-weaned piglets.

| Items                      | NC          | PC          | LP-ZnO      | HP-ZnO      | p-value |
|----------------------------|-------------|-------------|-------------|-------------|---------|
| Villus height (μm)         | 166.67±18.58 | 286.33±52.39 | 246.05±36.82 | 226.33±34.44 | 0.022   |
| Crypt depth (μm)           | 205.49±19.87 | 192.75±20.47 | 219.00±13.00 | 184.33±17.56 | 0.207   |
| Villus height/Crypt depth  | 0.87±0.16   | 1.45±0.25   | 1.31±0.26   | 1.22±0.22   | 0.067   |

1 Data shown as the mean±SD, n=6.

### Table 5. Effects of porous zinc oxide on serum concentration of biological parameters in early-weaned piglets.

| Items  | NC          | PC          | LP-ZnO      | HP-ZnO      | p-value |
|--------|-------------|-------------|-------------|-------------|---------|
| ALT (U/L) | 45.22±5.50 | 52.72±7.33  | 50.23±6.38  | 57.76±9.26  | 0.063   |
| AST (U/L) | 50.58±19.41 | 81.26±29.57 | 73.80±26.69 | 101.38±15.59 | 0.018   |
| Urea (mmol/L) | 3.28±1.21 | 4.02±1.32 | 3.24±1.22 | 3.84±0.83 | 0.795   |
| Cholesterol (mmol/L) | 0.43±0.15 | 0.46±0.16 | 0.49±0.13 | 0.42±0.08 | 0.782   |
| TP (g/L) | 39.88±1.36 | 40.10±6.19 | 40.65±3.19 | 41.77±2.86 | 0.832   |
| ALP (U/L) | 197.22±48.34 | 299.29±63.09 | 290.87±54.38 | 321.52±50.42 | 0.008   |
| GLU (mmol/L) | 5.96±1.21 | 2.83±0.92 | 3.47±1.47 | 2.28±0.57 | 0.000   |
| IgM (g/L) | 0.36±0.12 | 0.50±0.20 | 0.41±0.07 | 0.41±0.07 | 0.354   |
| IgG (g/L) | 1.52±0.43 | 2.73±0.38 | 2.07±0.80 | 2.55±0.61 | 0.041   |

1 Data shown as the mean±SD, n=6.

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Fig. 2. Effects of porous zinc oxide supplementation on the concentration of serum GH content (A), IGF-1 content (B) and Zn content (C) in the weaned piglets. Data were shown as the mean±SD, n=6. ab Mean values bearing different superscript letters were significantly different (p<0.05).
study \((p<0.05)\). Compared with the Control piglets, serum AST, ALP and IgG were higher in the other three groups, however, serum GLU concentration was decreased by 52.52%, 41.78% and 61.74%, respectively. As for the physiological parameters, there were significant differences in serum GH and IGF-1 concentrations among the four treatment groups (Fig. 2). Serum GH content in PC group was remarkably greater \((p<0.05)\) than that in LP-ZnO and HP-ZnO groups. Moreover, compared with the NC group, serum IGF-1 content in the HP-ZnO group were remarkably increased \((p>0.05)\). PC group had higher \((p<0.05)\) serum Zn content than NC and LP-ZnO group, but had no difference with HP-ZnO group (Fig. 2).

mRNA expression of inflammatory cytokines, tight junctions, aquaporin 3, and miR-122a in the jejunum of piglets

In this study, mRNA expression of inflammatory cytokines, tight junctions, aquaporin 3, and miR-122a in the jejunum of weaned piglets was determined. As shown in Fig. 3, jejunal mRNA abundance of TGF-β was significantly greater \((p<0.05)\) in the other three groups, while IL-8 expression was remarkably decreased \((p<0.05)\) by ZnO supplementation. No differences in TNF-α abundance was detected among the four treatments. In addition, jejunal Occludin and ZO-1 expressions were remarkably increased \((p<0.0001)\) by conventional ZnO or porous ZnO treatments (Fig. 4). However, according to the results of Fig. 5, piglets fed with 3,000 mg/kg of conventional ZnO as well as with 750
or 1,500 mg/kg of porous ZnO showed lower (p< 0.0001) mRNA levels for miR-122a and AQP3.

**DISCUSSION**

In pig industry, ZnO was widely used in piglet’s diet to decrease diarrhea rate and improve piglets’ growth performance (3–5, 8, 13). In this study, dietary supplementation with pharmacological dosage of ZnO (3,000 mg/kg) or porous ZnO (750, 1,500 mg/kg) remarkably increased ADG, while decreasing the diarrhea incidence and F/G ratio in weaned piglets. Meanwhile, the final BW and ADFI in the three ZnO treatment groups were numerically improved. Porous ZnO administration with 750 or 1,500 mg/kg displayed the comparable effect of in-feed regular ZnO (3,000 mg/kg) for enhancing piglet growth and decreasing diarrhea incidence, which indicated that the low levels of porous ZnO also played an effective role in promoting the growth performance in piglets.

Previous studies showed that high levels of dietary ZnO improved small intestinal morphology in weaned piglets (12, 24, 25), also associated with our results. In the current study, typically, piglets fed with porous ZnO underwent significant changes in the jejunal villus height comparing with the control group. These results indicated that the low levels of porous ZnO promoted small intestinal morphology. In addition, the current results showed no significant difference in the jejunal morphologic structure between porous ZnO (750 mg/kg, 1,500 mg/kg) and conventional ZnO (3,000 mg/kg) administration, suggesting that low level of porous ZnO exerted a similar effect on intestinal function compared with the ZnO.

Evidences have shown that serum biochemical and physiological parameters could indirectly response health status of weaned piglets (26). In the present study, results showed that piglets fed with porous ZnO (750 or 1,500 mg/kg) or conventional ZnO (3,000 mg/kg) present a greater concentration in the serum AST, ALP, and IgG than that in the control group, while showing lower serum GLU concentration. The reason may be that ZnO supplementation improved the adaptation of weaned piglets to immunological, nutritional, and psychological alterations. It is widely accepted that the high use of glucose maintains energy and health status of piglets and regulate the absorptive and transport capacity of various nutrition (27), including trace element Zn. Of note, although, the GH level in LP-ZnO and HP-ZnO groups was remarkably lower than that in PC group, but there was no difference compared with CN group, this may be only high doses of ZnO cans raise the GH level. But, we also found that dietary porous ZnO (1,500 mg/kg) supplementation could remarkably increase serum IGF-1, suggesting the improvement of porous ZnO in the growth and metabolism of weaned piglets. It is further confirmed by our study that porous ZnO at low doses as a substitute of regular ZnO (3,000 mg/kg), improved the health status of piglets in response to weaning transition. The limited Zn usage in pig industry can alleviate the environmental risk (28), thereby porous ZnO at low doses can decrease Zn pollution.

Early-weaned piglets are more susceptible to immune challenge due to their immature immune and digestive functions (29, 30). Especially, weaning is commonly triggering intestinal inflammation, that can be characterized by an upregulation of pro-inflammatory cytokines in the intestine (e.g., IL-1β, IL-6, and TNF-α) (31, 32). TGF-β is a multifunctional cytokine produced by many cell types including intestinal epithelia, which can improve intestinal integrity (33, 34). The cytokine IL-8 is considered one of the principal mediators in inflammation and it is a strong inducer of neutrophils (35). High dosage of ZnO is widely used to enhance resistance to inflammation, diarrhea and infection in weaned piglets (2, 36). In the current study, we found that supplementation with porous ZnO significantly upregulated the jejunal mRNA abundance of TGF-β and inhibited IL-8 expression, indicating that dietary supplementation with porous ZnO alleviated weaning stress-induced intestinal inflammation, especially under 1,500 mg/kg porous ZnO administration.

Weaning stress usually causes intestinal injury (i.e., barrier function and the changes in permeability) in piglets (37). Occludin and ZO-1 are two important tight junction proteins and mainly involve in intestinal permeability and barrier functions (38). In this study, 750 mg/kg or 1,500 mg/kg porous ZnO treatment significantly increased the mRNA expression of Occludin and ZO-1 in the jejunum of pigs compared with the piglets fed with 3,000 mg/kg regular ZnO. Moreover, low level of porous ZnO also markedly increased the mRNA expression of Occludin and ZO-1 in jejunal tissue. This indicates that porous ZnO at low doses could also increase intestinal barrier function for alleviating diarrhea in weaning piglets. In addition, defects in the intestinal epithelial tight junction barrier was attributed to intestinal inflammation and then results in the disorder of microRNA (miR)-122a and AQP3 mRNA expression (39, 40). Ye et al. (41) reported that the overexpressed miR-122a induced the degradation of Occludin mRNA and depletion of Occludin, leading to an increase in intestinal permeability. We showed that dietary ZnO supplementation decreased the mRNA expression of miR-122a and AQP3 in the jejunum of piglets, indicating that the ZnO supplementation might induce an increase in intestinal epithelial tight junction expression and it was regulated by a decrease in miR-122a expression in enterocytes. Ikarashi et al. (42) reported that intestinal AQP3 expression was markedly upregulated after administration of MgSO4, indicating that aquaporin might be highly associated with diarrhea, and our finding is similar to these results. However, the novel mechanism of miR-122a and AQP3 in the regulation of intestinal barrier function needs to be further warranted.

The present results indicate that porous ZnO supplemented diet at the doses of 750 or 1,500 mg/kg had the same effects as pharmacologic dose (3,000 mg/kg) of the conventional ZnO on promoting growth performance, intestinal morphology and health status (de-
increasing diarrhea incidence) in piglets after weaning, as well as positive effects on a mRNA expression of jejunal anti-inflammatory cytokine and tight junction genes, and decreasing the mRNA expression of pro-inflammatory factors, mir-122a and AQP3. This study also allowed to show that low doses of porous ZnO (750 mg/kg or 1,500 mg/kg) can be considered as an effective alternative to conventional regular ZnO (3,000 mg/kg) in promoting the intestinal physical and immune barrier function of weaned piglets. Thus, porous ZnO represents a reliable tool in the development of swine production systems for the reduction of post-weaning diarrhea, the subsequent prevention of gastrointestinal pathologies and the exportation of Zn to the environment.

Authorship

Research conception and design: PP, DD and RF; experiments: PP and SC; statistical analysis of the data: PP, CL, and JL; interpretation of the data: PP, AR, TL, and RF; writing of the manuscript: PP and XT.

Disclosure of state of COI

Peng Peng, Dun Deng, Sijia Chen, Chengliang Li, Jie Luo, Agathe Romeo, Tiejun Li, Xiaopeng Tang and Rejun Fang declare that they have no conflict of interest. The authors alone are responsible for the content and writing of this article.

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