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Diosmectite–zinc oxide composite improves intestinal barrier restoration and modulates TGF-β1, ERK1/2, and Akt in piglets after acetic acid challenge[1]

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ABSTRACT: The present study evaluated the beneficial effect of diosmectite–zinc oxide composite (DS-ZnO) on improving intestinal barrier restoration in piglets after acetic acid challenge and explored the underlying mechanisms. Twenty-four 35-d-old piglets (Duroc × Landrace × Yorkshire), with an average weight of 8.1 kg, were allocated to 4 treatment groups. On d 1 of the trial, colitis was induced via intrarectal injection of acetic acid (10 mL of 10% acetic acid [ACA] solution for ACA, DS-ZnO, and mixture of diosmectite [DS] and ZnO [DS+ZnO] groups) and the control group was infused with saline. Twenty-four hours after challenged, piglets were fed with the following diets: 1) control group (basal diet), 2) ACA group (basal diet), 3) DS-ZnO group (basal diet supplemented with DS-ZnO), and 4) DS+ZnO group (mixture of 1.5 g diosmectite [DS]/kg and 500 mg Zn/kg from ZnO [equal amount of DS and ZnO in the DS-ZnO treatment group]). On d 8 of the trial, piglets were sacrificed. The results showed that DS-ZnO supplementation improved ($P < 0.05$) ADG, ADFI, and transepithelial electrical resistance and decreased ($P < 0.05$) fecal scores, crypt depth, and fluorescein isothiocyanate-dextran 4kDa (FD4) influx as compared with ACA group. Moreover, DS-ZnO increased ($P < 0.05$) occludin, claudin-1, and zonula occluden-1 expressions; reduced ($P < 0.05$) caspase-9 and caspase-3 activity and Bax expression; and improved ($P < 0.05$) Bcl2, XIAP, and PCNA expression. Diosmectite–zinc oxide composite supplementation also increased ($P < 0.05$) TGF-β1 expression and ERK1/2 and Akt activation. These results suggest that DS-ZnO attenuates the acetic acid–induced colitis by improving mucosa barrier restoration, inhibiting apoptosis, and improving intestinal epithelial cells proliferation and modulation of TGF-β1 and ERK1/2 and Akt signaling pathway.

Key words: Akt, diosmectite–zinc oxide composite, ERK1/2, piglets, restoration, TGF-β1

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INTRODUCTION

Weaning is the most stressful phase in the life of pigs and is commonly associated with postweaning syndrome, which includes diarrhea, growth retardation, and intestinal barrier disruption (Boudry et al., 2004; Moeser et al., 2007; Smith et al., 2010; Peace et al., 2011). Disruption of the intestinal barrier results in the translocation of luminal antigens into subepithelial tissues (Blikslager et al., 2007), inciting mucosal and systemic inflammatory responses that play a central role in postweaning syndrome (Moeser et al., 2007; Smith et al., 2010). Therefore, improving the restoration of intestinal barrier may be beneficial in alleviating postweaning diarrhea.

It is well known that zinc is involved in resisting intestinal diseases. Numerous studies have demonstrated that zinc is involved in restoration of mucosal barrier (Roselli et al., 2003; Hu et al., 2014). Diosmectite (**DS**) has also been reported to reinforce the intestinal barrier and epithelial regeneration and it also acts as a carrier for various active ingredients (Hu et al., 2013a). However, ZnO has a higher solubility at acid pH. When

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ZnO in the diet reaches the stomach of piglets, most of the ZnO is transformed into Zn ions; therefore, just a small amount of ZnO can work in the intestine, which is the important site of its action. Zinc oxide in the diosmectite–zinc oxide composite (**DS-ZnO**) complex was intercalated between the interlayer spaces of DS, which could protect ZnO from being resolved in the stomach, thus increasing the percentage of ZnO molecules reaching the gastrointestinal tract and enhancing the efficiency of ZnO in the intestine. Our previous studies found that DS-ZnO prevented intestinal barrier disruption in weaning piglets (Hu et al., 2013a). We hypothesis that DS-ZnO might be effective in improve intestinal barrier restoration. However, little research has been conducted to investigate it before.

Acetic acid–induced colitis is a commonly used and easily inducible model to induce acute intestinal injury. Intrarectally injected acetic acid liberates protons within the intracellular space and causes massive intracellular acidification, resulting in epithelial damage and intestinal disorder. Recently, a porcine model of acetic acid–induced colitis have been developed and used to assess the beneficial effects of some nutritional substances on intestinal injury (Randhawa et al., 2014). TGF-β1, ERK1/2, and Akt signaling pathways are critical factors involved in mucosal restoration (El-Assal and Besner, 2005; Xiao et al., 2014). In the current study, we used this animal model to explore whether DS-ZnO supplementation could improve intestinal barrier restoration and influence TGF-β1, ERK1/2, and Akt signaling pathway. Additionally, a treatment of supplementing the mixture of DS and ZnO (equal amount of DS and ZnO in the DS-ZnO treatment) was used to compare the DS-ZnO's effects.

Materials and Methods

Animal Care and Experimental Design. This experiment was approved by the Animal Care and Use Committee, Zhejiang University (Hangzhou, Zhejiang, China). A total of twenty-four 35-d-old piglets (Duroc × Landrace × Yorkshire), with an average weight of 8.1 kg, were allocated to 4 treatment groups, each consisting of 6 animals. One group served as a control group and the other groups were subjected to the induction of ulcerative colitis by intrarectal injection with acetic acid. Experimental induction of ulcerative colitis was done according to the method described by Hou et al. (2014). Briefly, following 24 h of fasting (receiving nothing except water) and under anesthesia (with an intramuscular injection of sodium pentobarbital at a dose of 80 mg/kg BW), a soft catheter was introduced into the rectum (30–35 cm from the anus) for careful injection of acetic acid (10 mL of 10% acetic acid) or saline. Before removing the catheter, 20 mL air was applied to spread acetic acid into the colon. The dose of acetic acid and the distance of catheter into the anus were chosen according to our preliminary experiment and the studies of Hou et al. (2014). Twenty-four hours after colitis induction, each group was fed with the following diets: 1) control group (piglets fed the basal diet), 2) acetic acid group (piglets fed the basal diet), 3) DS-ZnO group (piglets fed the basal diet supplemented with the DS-ZnO [500 mg Zn/kg] diet), and 4) mixture of diosmectite (**DS**) and ZnO (**DS+ZnO**; mixture of 1.5 g DS/kg and 500 mg Zn/kg from ZnO [equal amount of DS and ZnO in the DS-ZnO treatment group]). Diets were formulated to meet or exceed requirements suggested by the NRC (1998; Table 1). The piglets were given ad libitum access to feed and water. After the trial, ADG, ADFI, and feed conversion efficiency were calculated. Fecal scores were visually assessed every day on a 5-point scale ranging from 1 to 5 according to the method of Hu et al. (2013b): 1 = no diarrhea, hard feces; 2 = no diarrhea, normal consistency of feces; 3 = mild diarrhea, soft and partially formed feces; 4 = moderate diarrhea, semiliquid feces; and 5 = severe diarrhea, watery feces.

Sample Collection. On d 8 of the trial, piglets were humanely killed and 1 intestinal segment measuring

Table 1. Ingredient composition of the basal diet (on an as-fed basis)

Ingredient, g/kg	Content, g/kg
Maize	560
Soybean meal	294
Fish meal	55
Dried whey	45
Soybean oil	15
Limestone meal	5
Dicalcium phosphate	11.5
Sodium chloride	3
L-Lysine HCl	1
DL-Methionine	0.5
Vitamin–mineral premix[1]	10
Analyzed composition, g/kg	
Digestible energy,[2] MJ/kg	14.6
CP	221.8
Lysine	15.4
Methionine	3.7
Calcium	9.0
Total phosphorus	7.8
Zn, mg/kg	132.3

[1]Provided per kilogram of diet: 6,000 IU vitamin A, 500 IU vitamin D_3, 40 IU vitamin E, 20 mg pantothenic acid, 30 mg niacin, 5.0 mg riboflavin, 1.5 mg vitamin K_3, 2.0 mg thiamine, 3.0 mg pyridoxine, 0.10 mg biotin, 0.6 mg folic acid, 0.04 mg vitamin B_{12}, 800 mg choline, 16 mg Cu ($CuSO_4 \cdot 5H_2O$), 100 mg Zn ($ZnSO_4$), 125 mg Fe ($FeSO_4$), 15 mg Mn ($MnSO_4 \cdot H_2O$), 0.2 mg I (KI), and 0.3 mg Se (Na_2SeO_3).

[2]Digestible energy was calculated from data provided by Feed Database in China (2012).

3 cm in length and 2 measuring 10 cm were excised from the distal colon. The 3-cm intestinal segments were flushed with ice-cold PBS and then fixed in 10% fresh, chilled formalin solution. One 10-cm intestinal segment was immediately placed into Ringer's solution and mounted in Ussing chambers, as described by Hu et al. (2013b). The other 10-cm intestinal segment was opened longitudinally and the contents were flushed with ice-cold PBS. The mucosa samples were harvested by scraping with a glass slide, immediately frozen in liquid nitrogen, and then stored at –80°C for further analysis. The frozen mucosa samples were weighed, homogenized, and centrifuged (3000 × g ,10 min, 4°) to collect the supernatant according to Hu et al. (2014). The supernatant was used for analysis.

Intestinal Morphology and Barrier Function. After a 24-h fixation, the intestinal segments were dehydrated, embedded, and stained with hematoxylin and eosin. Crypt depth was measured in at least 10 well-oriented crypt units using image analysis (Leica Imaging Systems Limited, Cambridge, UK) and averaged for each sample. The transepithelial electrical resistance (**TER**) and mucosal-to-serosal permeability to 4 kDa fluorescein isothiocyanate–dextran (**FD4**; Sigma-Aldrich, St. Louis, MO) were determined in vitro in the Ussing chamber, according to the procedures outlined by Hu et al. (2013b). Briefly, segments of colon were stripped from the seromuscular layer in oxygenated (95% O_2/5% CO_2) Ringer's solution and then mounted in the EasyMount Ussing chamber system (Physiologic Instruments, San Diego, CA) with a multichannel voltage-current clamp (model VCC MC6; Physiologic Instruments, San Diego, CA). The clamps were connected to acquire and analyze software (Physiologic Instruments) for automatic data collection. After a 30-min equilibration period on the Ussing chambers, TER ($\Omega \cdot cm^2$) was recorded at 15-min intervals over a 2-h period and then averaged to derive the TER values for a given pig. Fluorescein isothiocyanate–dextran (Sigma-Aldrich) was added on the mucosal side at a final concentration of 0.375 mg/mL. Mucosal-to-serosal flux of FD4 ($\mu g \cdot cm^{-2} \cdot h^{-1}$) was monitored from the serosal side at 30-min intervals for 120 min. The concentrations of FD4 in the serosal side were measured by fluorescence microplate reader (FLx800; Bio-Tek Instruments Inc., Winooski, VT). The flux over the 2-h period was calculated.

Caspase Activity Assays. Caspase activity in colon mucosa was determined using a caspase activity kit according to the manufacturer's instructions (Beyotime Institute of Biotechnology, Jiangsu, China). Caspase activities were expressed as the percentage of enzyme activity compared with the control.

Protein Expression Analysis by Western Blot. Western blot analysis was performed according to the procedures outlined by Hu et al. (2013b). Briefly, after electrophoresis, the proteins were transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membranes were incubated overnight at 4°C with the primary antibody (**Ab**) and then with the secondary Ab for 120 min at room temperature. The primary Ab (occludin, claudin-1, zonula occluden-1 [**ZO-1**], Bax, Bcl2, X-linked inhibitor of apoptosis protein [**XIAP**], proliferating cell nuclear antigen [**PCNA**], transforming growth factor-β1 [**TGF-β1**], β-tublin, and phospho-ERK1/2 rabbit mAb) were purchased from Santa Cruz Technology Inc. (Danvers, MA). The secondary Ab was alkaline phosphatase–conjugated anti-rabbit Ab (Sigma-Aldrich). The immunoreactive protein bands on the membrane were developed according to the methods of Wang et al. (2013). The western blotting result was evaluated using Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA). The β-tublin was used as an internal control, which exhibited no difference within each group. The relative abundance of each target protein was expressed as target protein:β-tublin protein ratio. The protein expression of all samples was expressed as fold changes, calculated relative to the control group.

Table 2. Effects of diosmectite–zinc oxide composite (DS-ZnO) supplementation on growth performance and fecal scores of piglets[1]

Item	Control	Acetic acid	DS-ZnO[1]	DS+ZnO[2]	SEM[3]	*P*-value
ADG, g	298.83[a]	234.17[c]	281.67[ab]	247[bc]	11.57	0.003
ADFI, g	519.67[a]	434[c]	497.83[ab]	459.83[bc]	18.72	0.019
Feed:gain	1.75	1.87	1.77	1.87	0.07	0.513

[a–c]Within a row, means without a common superscript differ ($P < 0.05$).

[1]Containing 250 g Zn/kg DS-ZnO.

[2]DS+ZnO = mixture of diosmectite (DS) and ZnO. Supplemental mixture of diosmectite and ZnO (equal amount of diosmectite and ZnO in the DS-ZnO group).

[3]$n = 6$.

Statistical Analysis

Data were analyzed using the SAS statistical package (SAS Inst. Inc., Cary, NC), with each animal considered an experimental unit. Differences between the other means were tested using Duncan's multiple range tests. Differences were considered significant at $P < 0.05$.

RESULTS

Growth Performance and Fecal Scores

Table 2 shows the growth performance of piglets. Compared with the control group, the acetic acid group

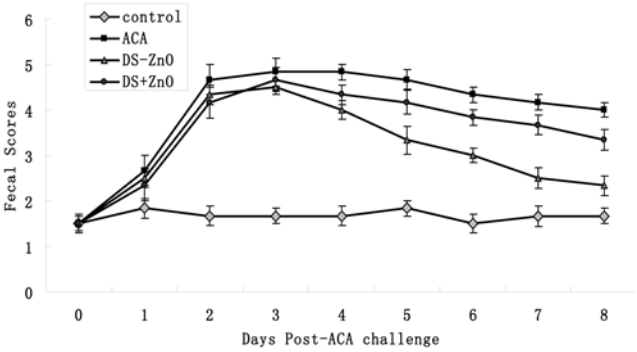

Figure 1. Effect of diosmectite–zinc oxide composite (DS-ZnO) supplementation on fecal scores of piglets. Weaned pigs were challenged with acetic acid (d 0) and fecal scores were recorded daily over an 8-d experimental period. Data represent means ± SE (n = 6). Acetic acid challenge increased fecal scores in all acetic acid–challenged group (acetic acid group, DS-ZnO group, and mixture of diosmectite (DS) and ZnO [DS+ZnO] group) compared with control group after d 2. Diosmectite–zinc oxide composite contained 250 g zinc/kg DS-ZnO. ACA = acetic acid; DS+ZnO = mixture of 1.5 g diosmectite (DS)/kg and 500 mg Zn/kg from ZnO (equal amount of DS and ZnO in the DS-ZnO treatment group)

exhibited a reduction ($P < 0.05$) in ADG and ADFI. Dietary supplementation of DS-ZnO improved ($P < 0.05$) ADG and ADFI compared with the acetic acid group, whereas supplementation with the mixture of DS and ZnO did not ($P > 0.05$) have these effects. Before acetic acid challenge, there was no indication of diarrhea ($P > 0.05$) in any of the pigs in this study (Fig. 1). Acetic acid challenge increased fecal scores ($P < 0.05$) in all acetic acid–challenged groups (acetic acid group, DS-ZnO group, and DS+ZnO group) compared with control group after d 2. There was no significant difference ($P > 0.05$) in fecal scores from d 2 to 4 among all acetic acid–challenged groups, whereas after d 4, the fecal scores in the DS-ZnO group were significantly lower than the acetic acid group and the DS+ZnO group ($P < 0.05$).

Table 3. Effects of diosmectite–zinc oxide composite (DS-ZnO) supplementation on the colon morphology and barrier function of piglets

Item	Control	Acetic acid	DS-ZnO[1]	DS+ZnO[2]	SEM[3]	*P*-value
Crypt depth, μm	343.67^{c}	373.17^{a}	355.83^{bc}	362.00^{ab}	5.15	<0.005
TER,[4] Ω·cm^2	82.83^{a}	51.83^{b}	74.33^{a}	59.00^{b}	3.61	<0.001
FD4[5] flux, μg·cm^{-2}·h^{-1}	0.31^{c}	0.58^{a}	0.34^{c}	0.49^{b}	0.02	<0.001

$^{a–c}$Within a row, means without a common superscript differ ($P < 0.05$).

[1]Containing 250 g Zn/kg DS-ZnO.

[2]DS+ZnO = mixture of diosmectite (DS) and ZnO. Supplemental mixture of diosmectite and ZnO (equal amount of diosmectite and ZnO in the DS-ZnO group).

[3]n = 6.

[4]TER = transepithelial electrical resistance.

[5]FD4 = fluorescein isothiocyanate–dextran.

Colon Morphology and Barrier Function

Table 3 shows the colon morphology and barrier function of piglets. As compared with control group, the pigs challenged with acetic acid had higher ($P < 0.05$) crypt depth and FD4 flux and lower ($P < 0.05$) TER in the colon of piglets. Piglets fed with DS-ZnO had higher ($P < 0.05$) TER and lower ($P < 0.05$) crypt depth and FD4 flux compared to acetic acid group. The supplemental mixture of DS and ZnO decreased ($P < 0.05$) the FD4 flux but did not affect ($P > 0.05$) the crypt depth and TER of colon compared with acetic acid group.

Tight Junction Protein Expression

Figure 2 shows the protein expression of occludin, claudin-1, and ZO-1 in colonic mucosa of piglets. As compared with the control group, acetic acid challenge

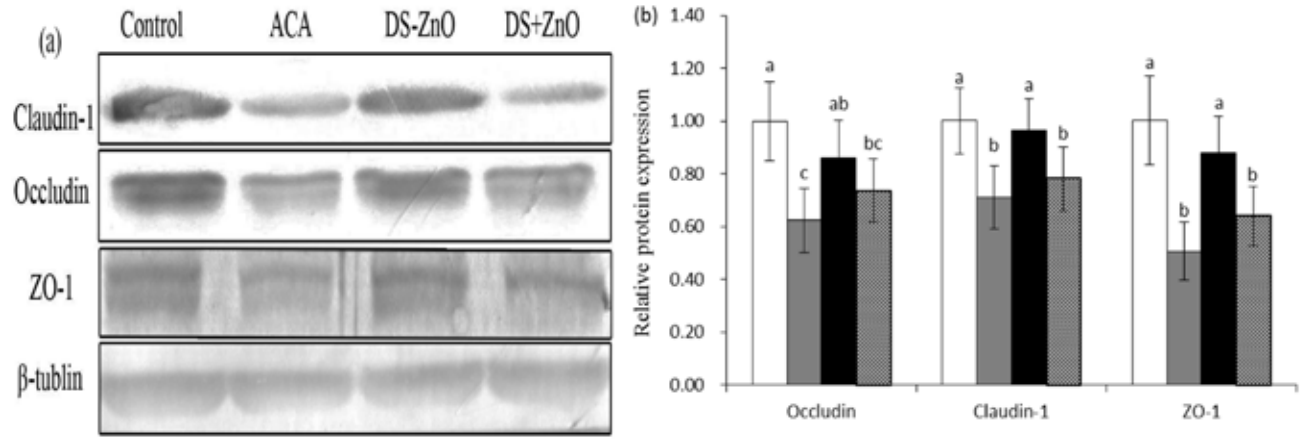

Figure 2. Effects of diosmectite–zinc oxide composite (DS-ZnO) supplementation on protein expression of occludin, claudin-1, and zonula occluden-1 (ZO-1) in colonic mucosa of piglets. Panel (a) shows representative blots of occludin, claudin, ZO-1, and β-tublin in colonic mucosa of piglets. Panel (b) shows relative tight junction proteins expression in colonic mucosa of piglets. Values are means and SD represented by vertical bars. ^{a,b,c}Means with different letters differ significantly ($P < 0.05$). Diosmectite–zinc oxide composite contained 250 g zinc/kg DS-ZnO. ACA = acetic acid; DS+ZnO = mixture of diosmectite (DS) and ZnO (equal amount of DS and zinc oxide in the DS-ZnO group). The control sample was used as the reference sample. The protein expression of all samples was expressed as fold changes, calculated relative to the control group. □ = control; ▒ = acetic acid; ■ = DS-ZnO; ▒ = DS+ZnO.

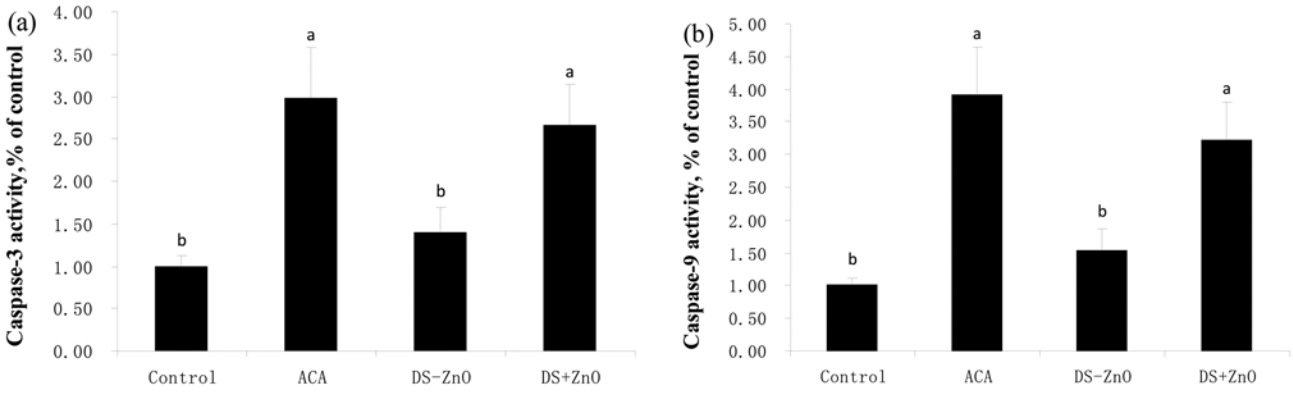

Figure 3. Effects of diosmectite–zinc oxide composite (DS-ZnO) supplementation on activity of caspases-9 and caspase-3. Caspase-3 (a) and caspase-9 (b) activation were measured using the chromogenic substrates Ac-LEHD-pNA(acetyl-Leu-Glu-His-Asp p-nitroanilide) and Ac-LEHD-pNA(N-Acetyl-Asp-Glu-Val-Asp p-nitroanilide), respectively. Values are means and SD represented by vertical bars. [a,b,c]Means with different letters differ significantly ($P < 0.05$). Diosmectite–zinc oxide composite contained 250 g zinc/kg DS-ZnO. ACA = acetic acid; DS+ZnO = mixture of diosmectite (DS) and ZnO (equal amount of DS and zinc oxide in the DS-ZnO group). The control sample was used as the reference sample. The value was expressed as fold changes relative to the control animals.

decreased ($P < 0.05$) protein expression of occludin, claudin-1, and ZO-1. The piglets fed with DS-ZnO at 500 mg Zn/kg had higher ($P < 0.05$) occludin, claudin-1, and ZO-1 protein expression compared to the acetic acid group. Supplementation with the mixture of DS and ZnO did not affect ($P > 0.05$) the tight junction (**TJ**) expression compared with acetic acid group.

Caspase Activity

Figure 3 shows the activity of caspase-9 and caspase-3 in colonic mucosa of piglets. Compared to the control group, piglets challenged with acetic acid had a higher ($P < 0.05$) caspase-9 and caspase-3 activity. Dietary supplementation with DS-ZnO decreased ($P < 0.05$) the activity of caspase-9 and caspase-3 as compared with the acetic acid group. Supplementation with the mixture of DS and ZnO did not ($P > 0.05$) affect the activity of caspase-9 and caspase-3 in comparison with the acetic acid group.

Apoptosis-Related Protein and PCNA Expression

Figure 4 shows the expression of apoptosis-related protein and PCNA expression in colonic mucosa of piglets. Compared with the control group, acetic acid challenge increased ($P < 0.05$) Bax expression and decreased ($P < 0.05$) Bcl2, XIAP, and PCNA expression. Supplementation with DS-ZnO increased ($P < 0.05$) the expression of Bcl2, XIAP, and PCNA and decreased ($P < 0.05$) Bax expression compared with the acetic acid group. Supplementation with the

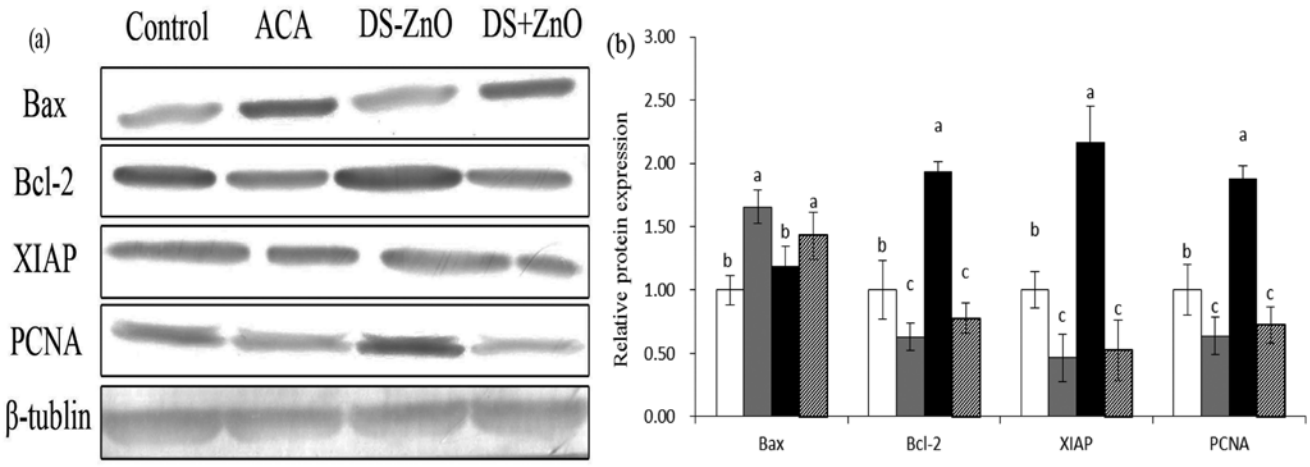

Figure 4. Effects of diosmectite–zinc oxide composite (DS-ZnO) supplementation on the protein expression of Bax, Bcl-2, XIAP, and PCNA in colonic mucosa of piglets. Panel (a) shows representative blots of Bax, Bcl-2, XIAP, PCNA, and β-tublin in colonic mucosa of piglets. Panel (b) shows relative proteins expression in colonic mucosa of piglets. Values are means and SD represented by vertical bars. [a,b,c]Means with different letters differ significantly ($P < 0.05$). Diosmectite–zinc oxide composite contained 250 g zinc/kg DS-ZnO. ACA = acetic acid; DS+ZnO = mixture of diosmectite (DS) and ZnO (equal amount of DS and zinc oxide in the DS-ZnO group). The control sample was used as the reference sample. The protein expression of all samples was expressed as fold changes, calculated relative to the control group. □ = control; ▒ = acetic acid; ■ = DS-ZnO; ▨ = DS+ZnO.

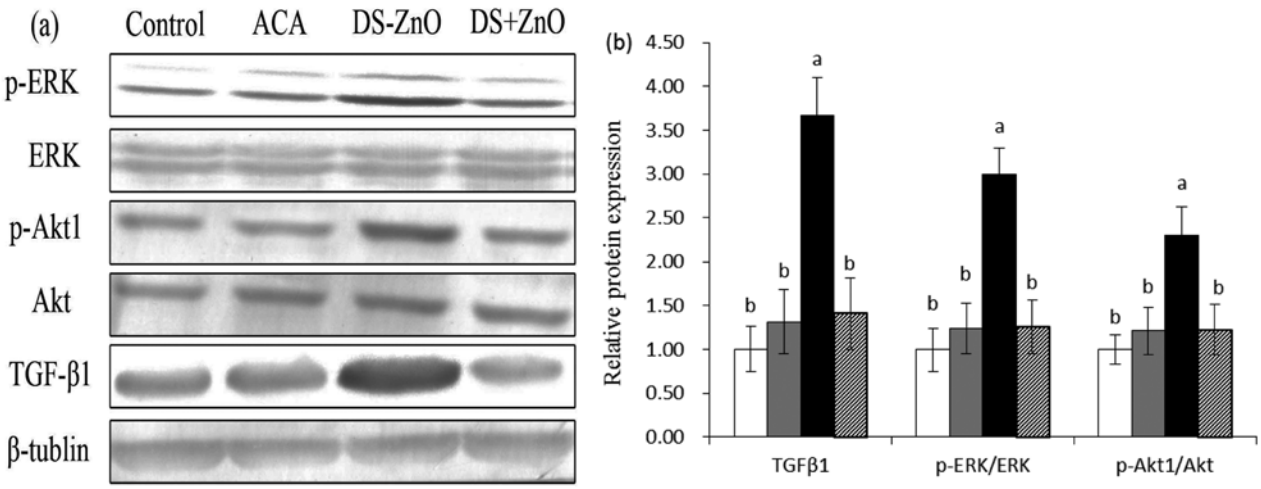

Figure 5. Effects of diosmectite–zinc oxide composite (DS-ZnO) supplementation on the protein expression of TGF-β1 and the activation of ERK and Akt in colonic mucosa of piglets. Panel (a) shows representative blots of TGF-β1, ERK, Akt, and β-tublin in colonic mucosa of piglets. Panel (b) shows relative proteins expression in colonic mucosa of piglets. Values are means and SD represented by vertical bars. [a,b,c]Means with different letters differ significantly ($P < 0.05$). Diosmectite–zinc oxide composite contained 250 g zinc/kg DS-ZnO. ACA = acetic acid; DS+ZnO = mixture of diosmectite (DS) and ZnO (equal amount of DS and zinc oxide in the DS-ZnO group). The control sample was used as the reference sample. The protein expression of all samples was expressed as fold changes, calculated relative to the control group. □ = control; ▒ = acetic acid; ■ = DS-ZnO; ▨ = DS+ZnO.

mixture of DS and ZnO had no effect ($P > 0.05$) on the expression of Bcl2, XIAP, and PCNA expression compared with acetic acid group.

TGF-β1 Expression

Figure 5 shows the expression of TGF-β1 expression in colonic mucosa of piglets. Piglets challenged with acetic acid did not differ ($P > 0.05$) from the control group on TGF-β1 expression. Dietary supplementation with DS-ZnO increased ($P < 0.05$) the expression of TGF-β1 compared with the acetic acid group and control group. Supplemented with the mixture of DS and ZnO do not affected ($P > 0.05$) the expression of TGF-β1 induced by acetic acid.

ERK and PI3K/Akt Signal Pathway

Figure 5 shows the activation of the ERK and Akt signaling pathways in colonic mucosa of piglets. As compared with the control group, acetic acid challenge did not ($P > 0.05$) significantly activate the ERK and Akt pathway. Supplementation with DS-ZnO improved ($P < 0.05$) the activation of ERK and AKT compared to acetic acid groups and the control group. Supplementation with the mixture of DS and ZnO had no effect ($P > 0.05$) on the activation of ERK and Akt signaling pathways compared with acetic acid group.

DISCUSSION

In the present study, pigs challenged with acetic acid showed significantly lower ADG and ADFI and higher fecal scores, whereas DS-ZnO supplementation improved ADG and ADFI and decreased the fecal scores compared with acetic acid–challenged piglets. This increased feed intake might have beneficial effects on the intestinal injury. McCracken et al. (1999) reported that depressed feed intake leads to local inflammation and atrophy of the mucosa in the piglet small intestine and that intestinal inflammation subsides and epithelial morphology improves when normal feed intake patterns resume. Feed intake increase might provide sufficient energy and nutrients for the epithelium restoration and indirectly improve the intestinal barrier function. The change of colon morphology and barrier function also confirmed the beneficial effect of DS-ZnO on improving intestinal restoration. In the current experiment, the acetic acid challenge increased colon crypt depth and FD4 flux and decreased TER, whereas supplementation with DS-ZnO reduced crypt depth and FD4 flux and increased TER in colon of piglets. However, a supplemental mixture of DS and ZnO in the present experiment had no influence on acetic acid–challenged crypt depth and intestinal barrier function. This may result from the amount of DS (1.5 g/kg diet) and ZnO (500 mg/kg diet) supplemented in this study being too low, as it was reported that the beneficial effect of DS was dose dependent and suggested to be 20 to 30 g/kg and the effective addition of ZnO should up to 2,000 mg/kg in weaning pigs (Hollis et al., 2005; Song et al., 2012; Hu et al., 2013a). Diosmectite can delay and/or target drug release, increase drug stability, and modify drug delivery patterns, such as vitamin B_6 (Joshi et al., 2009). The different effects between the mixture of DS and ZnO and the DS-ZnO complex on

gut responses in this study indicated that the beneficial effects of DS-ZnO might depend on its unique properties that are not due to the properties of ZnO or DS. Zinc oxide has a higher solubility at acid pH and the ZnO molecule itself plays a crucial role in alleviating the incidence of diarrhea. When ZnO in the diet passes the stomach of piglets, most of the ZnO is transformed into Zn ions; therefore, just a small amount of ZnO can work in the intestine, which is the important site of its action. If ZnO is processed to make its dissociation percentage lower in the stomach, a larger amount of ZnO can reach the intestine; therefore, a lower dose of ZnO could be used in the diet (Hu et al., 2013c). The beneficial effects of diosmectite on the intestine were also dose dependent and suggested to be 20 to 30 g/kg. However, ZnO in the DS-ZnO complex was intercalated between the interlayer spaces of DS (Khaorapapong et al., 2011; Hu et al., 2012a). This could protect ZnO from being dissolved in the stomach and markedly increase the percentage of the ZnO molecule reaching the gastrointestinal tract and enhance the efficiency of ZnO in the intestine. Our previous study found that supplementation with DS-ZnO at 500 mg Zn/kg was effective in protecting intestinal mucosal barrier and had better performance than the equivalent amount of mixture of DS and ZnO (Hu et al., 2012b, 2013a). According to the above results, we speculated that enhancing the restoration of epithelium after injury might be another mechanism that DS-ZnO protects the intestinal integrity and barrier function in an acetic acid–induced colitis model.

The TJ is a major cellular component for maintenance of tissue integrity and barrier function. It has a complex molecular composition and includes the transmembrane protein complexes (e.g., claudins and occludins) and the cytosolic proteins ZO (e.g., junctional adhesion molecule, ZO-1, ZO-2, and ZO-3). These proteins form a structure at the boundary of 2 adjacent cells, working as a barrier within the epithelial cell space. There is evidence showing that intestinal TJ barrier dysfunction can accelerate the onset and severity of postweaning diarrhea (Smith et al., 2010; Hu et al., 2014). In our current study, consistent with the improved intestinal integrity, DS-ZnO supplementation increased the expression of occludin, claudin-1, and ZO-1. Similarly, our previous study in weaning piglets also showed that supplementation with DS-ZnO increased protein expression levels of occludin, claudin-1, and ZO-1 (Hu et al., 2013a). Consequently, in the present study, DS-ZnO may partially improve the intestinal integrity and barrier function via increasing the TJ proteins expression.

The dynamic balance between intestinal epithelial cell (**IEC**) proliferation and apoptosis is another critical factor involved in maintaining the intestinal integrity. Intestinal epithelial cell apoptosis can disrupt intestinal mucosal integrity and has been detected in weaning piglets (Zhu et al., 2014). Caspase-9 and caspase-3 are common indicators that reflect cell apoptosis and are negatively correlated with intestinal integrity and barrier function (Liu et al., 2012). In our study, acetic acid challenge increased the activities of caspase-9 and caspase-3, whereas DS-ZnO decreased the activities of caspase-9 and caspase-3, suggesting that DS-ZnO probably attenuated acetic acid–induced intestinal injury by inhibiting IEC apoptosis. In line with our results, Liang et al. (2012b) reported that zinc suppressed the activation of caspase-3 and inhibits apoptosis. Bax, Bcl2, and XIAP are also important indicators for apoptosis. In the current study, the acetic acid challenge increased the Bax expression and decreased the Bcl2 and XIAP expression, confirming that DS-ZnO reduced acetic acid–induced intestinal injury by inhibiting apoptosis. Similar to our findings, Zhang et al. (2014) found that zinc reduced the Bax expression and inhibited high glucose-induced apoptosis in renal tubular epithelial cells. Hao et al. (2014) found that zinc reduced the Bax expression and increased the Bcl-2 expression in a depleted uranium–induced apoptosis. In addition, we found that piglets fed with DS-ZnO had a higher expression of PCNA, a marker of proliferation, in colon mucosa as compared with the acetic acid–challenged group, suggesting that DS-ZnO stimulated the regeneration of IEC. Similar to our results, Seo et al. (2010) found that zinc increased bone formation through stimulating cell proliferation in osteoblastic MC3T3-E1 cells. In the current study, feeding pigs with a DS-ZnO diet may improve intestinal barrier restoration partially by inhibiting apoptosis and stimulating cell proliferation.

According to our above findings, DS-ZnO improves intestinal barrier restoration after acetic acid challenge. TGF-β is considered to be the key regulator of intestinal injury restoration as most of the cytokines that promote epithelial restitution act through a TGF-β-dependent mechanism (Xiao et al., 2014). Andújar et al. (2013) found that TGF-β positively regulates gastrointestinal ulcer healing. Xiao et al. (2014) showed that the decline in TGF-β1 level is closely related to the intestinal barrier dysfunction in weaning piglets. In our current experiment, the TGF-β1 expression in colon mucosa was increased in piglets supplemented with DS-ZnO compared with the acetic acid–challenged pigs. Consistent with our findings, zinc markedly increased the concentration of TGF-β1 in the culture medium secreted from osteoblastic MC3T3-E1 cells (Yamaguchi and Hashizume, 1994). In femoral tissues of newborn rats and bone tissues with fracture healing, zinc has a stimulatory effect on TGF-β1 production (Igarashi and Yamaguchi, 2001; Ma et al.,

2001). In human Caco-2 enterocytes, Roselli et al. (2003) showed that ZnO upregulated TGF-β mRNA in *Escherichia coli* K88–infected cells.

The ERK1/2 cascade is involved in cell growth and differentiation and is activated during healing of gastrointestinal epithelium by growth factors both in vivo and in vitro (El-Assal and Besner, 2005; Hu et al., 2013b). Recently, it has been reported that the ERK signaling pathway is involved in the regulation of TJ expression and the integrity of the epithelial barrier (Hu et al., 2013b; Song et al., 2014). The involvement of ERK1/2 in mucosal recovery also has been shown (Shifflett et al., 2004; El-Assal and Besner, 2005). In the present study, supplementation with DS-ZnO improved the activation of ERK as compared with the acetic acid groups. In agreement, Liang et al. (2012a) reported that zinc increased osteogenic function in mouse MC3T3-E1 osteoblasts through stimulating ERK signaling pathways. Wang et al. (2009) reported that supplementation with 3,000 mg Zn/kg from ZnO increased A-Raf-1 expression in the jejunum of weaning piglets, which is a key member of the Ras/Raf/MEK/ERK signaling pathway. There is also some evidence that the activation of ERK1/2 signaling is linked to the TGF-β1-induced modulation of TJ permeability. In cultured rat IEC (IEC-6), TGF-β1 increased wound closures via ERK activation (Suer et al., 2009). TGF-β1 stimulated re-epithelialization in human corneal epithelial cells through the Ras/MEK/ERK signaling pathway (Secker et al., 2008). In our study, it is possible that the effects of DS-ZnO on intestinal integrity recovery are also related to activation of ERK1/2.

Besides the ERK signaling pathway, Akt is another key factor involved in intestinal injury restoration. Numerous studies reported that activated Akt promotes the survival of IEC**]** and increases the tolerance of tissue to injuries via inhibition of various apoptotic pathways (El-Assal and Besner, 2005; Liang et al., 2012b). El-Assal and Besner (2005) reported that blocking of PI3K/Akt resulted in a significant reduction in spontaneous restitution. In the current study, supplementation with DS-ZnO enhanced activation of Akt as compared with the acetic acid–challenged group and was related to recovery of intestinal injury. Consistent with our results, Liang et al. (2012b) reported that zinc inhibits H_2O_2–induced MC3T3-E1 cell apoptosis via MAPK and PI3K/AKT pathways. The results of the current study indicate that the activation of the Akt signaling pathway was involved in the effect of DS-ZnO on intestinal injury restoration.

In summary, the present study demonstrated that DS-ZnO supplementation exerts beneficial effect in improving intestinal barrier restoration and modulating the expression of TJ proteins, inhibiting the apoptosis and enhancing proliferation of epithelial cells. TGF-β, ERK1/2, and Akt signaling pathways could be critical for the beneficial effect of DS-ZnO on improving intestinal barrier restoration.

LITERATURE CITED

Andújar, I., J. L. Ríos, R. M. Giner, and M. C. Recio. 2013. Shikonin promotes intestinal wound healing in vitro via induction of TGF-β release in IEC-18 cells. Eur. J. Pharm. Sci. 49:637–641. doi:10.1016/j.ejps.2013.05.018.

Blikslager, A. T., A. J. Moeser, J. L. Gookin, S. L. Jones, and J. Odle. 2007. Restoration of barrier function in injured intestinal mucosa. Physiol. Rev. 87:545–564. doi:10.1152/physrev.00012.2006.

Boudry, G., V. Péron, I. Le Huërou-Luron, J. P. Lallès, and B. Sève. 2004. Weaning induces both transient and long-lasting modifications of absorptive, secretory, and barrier properties of piglet intestine. J. Nutr. 134:2256–2262.

El-Assal, O. N., and G. E. Besner. 2005. HB-EGF enhances restitution after intestinal ischemia/reperfusion via PI3K/Akt and MEK/ERK1/2 activation. Gastroenterology 129:609–625. doi:10.1053/j.gastro.2005.05.054.

Feed Database in China. 2012. Table of Feed Composition and Nutritive Value in China. 23rd ed. China Feed, Beijing, China.

Hao, Y., J. Ren, C. Liu, H. Li, J. Liu, Z. Yang, R. Li, and Y. Su. 2014. Zinc protects human kidney cells from depleted uranium-induced apoptosis. Basic Clin. Pharmacol. 114:271–280. doi:10.1111/bcpt.12167.

Hollis, G. R., S. D. Carter, T. R. Cline, T. D. Crenshaw, G. L. Cromwell, G. M. Hill, S. W. Kim, A. J. Lewis, D. C. Mahan, P. S. Miller, H. H. Stein, and T. L. Veum. 2005. Effects of replacing pharmacological levels of dietary zinc oxide with lower dietary levels of various organic zinc sources for weanling pigs. J. Anim. Sci. 83:2123–2129.

Hou, Y., L. Wang, D. Yi, B. Ding, X. Chen, Q. Wang, H. Zhu, Y. Liu, Y. Yin, J. Gong, and G. Wu. 2014. Dietary supplementation with tributyrin alleviates intestinal injury in piglets challenged with intrarectal administration of acetic acid. Br. J. Nutr. 111:1748–1758. doi:10.1017/S0007114514000038.

Hu, C. H., L. Y. Gu, Z. S. Luan, J. Song, and K. Zhu. 2012a. Effects of montmorillonite–zinc oxide hybrid on performance, diarrhea, intestinal permeability and morphology of weanling pigs. Anim. Feed Sci. Technol. 177:108–115. doi:10.1016/j.anifeedsci.2012.07.028.

Hu, C., J. Song, Y. Li, Z. Luan, and K. Zhu. 2013a. Diosmectite-zinc oxide composite improves intestinal barrier function, modulates expression of pro-inflammatory cytokines and tight junction protein in early weaned pigs. Br. J. Nutr. 110:681–688. doi:10.1017/S0007114512005508.

Hu, C. H., Z. H. Song, K. Xiao, J. Song, L. F. Jiao, and Y. L. Ke. 2014. Zinc oxide influences intestinal integrity, the expressions of genes associated with inflammation and TLR4-myeloid differentiation factor 88 signaling pathways in weanling pigs. Innate Immun. 20:478–486. doi:10.1177/1753425913499947.

Hu, C. H., J. Song, Z. T. You, Z. S. Luan, and W. F. Li. 2012b. Zinc oxide-montmorillonite hybrid influences diarrhea, intestinal mucosal integrity and digestive enzyme activity in weaned pigs. Biol. Trace Elem. Res. 149:190–196. doi:10.1007/s12011-012-9422-9.

Hu, C. H., K. Xiao, Z. S. Luan, and J. Song. 2013b. Early weaning increases intestinal permeability, alters expression of cytokine and tight junction proteins, and activates mitogen-activated protein kinases in pigs. J. Anim. Sci. 91:1094–1101. doi:10.2527/jas.2012-5796.

Hu, C. H., K. Xiao, J. Song, and Z. S. Luan. 2013c. Effects of ZnO supported on zeolite on growth performance, intestinal microflora and permeability, and cytokines expression of weaned pigs. Anim. Feed Sci. Technol. 181:65–71. doi:10.1016/j.anifeedsci.2013.02.003.

Igarashi, A., and M. Yamaguchi. 2001. Increase in bone growth factors with healing rat fractures: The enhancing effect of zinc. Int. J. Mol. Med. 8:433–438.

Joshi, G. V., B. D. Kevadiya, H. A. Patel, H. C. Bajaj, and R. V. Jasra. 2009. Montmorillonite as a drug delivery system: Intercalation and in vitro release of timolol maleate. Int. J. Pharm. 374:53–57. doi:10.1016/j.ijpharm.2009.03.004.

Khaorapapong, N., N. Khumchoo, and M. Ogawa. 2011. Preparation of zinc oxide-montmorillonite hybrids. Mater. Lett. 65:657–660. doi:10.1016/j.matlet.2010.11.052.

Liang, D., M. Yang, B. Guo, J. Cao, L. Yang, and X. Guo. 2012a. Zinc upregulates the expression of osteoprotegerin in mouse osteoblasts MC3T3-E1 through PKC/MAPK pathways. Biol. Trace Elem. Res. 146:340–348. doi:10.1007/s12011-011-9254-z.

Liang, D., M. Yang, B. Guo, J. Cao, L. Yang, X. Guo, Y. Li, and Z. Gao. 2012b. Zinc inhibits H_2O_2–induced MC3T3-E1 cells apoptosis via MAPK and PI3K/AKT pathways. Biol. Trace Elem. Res. 148:420–429. doi:10.1007/s12011-012-9387-8.

Liu, Y., F. Chen, J. Odle, X. Lin, S. K. Jacobi, H. Zhu, Z. Wu, and Y. Hou. 2012. Fish oil enhances intestinal integrity and inhibits TLR4 and NOD2 signaling pathways in weaned pigs after LPS challenge. J. Nutr. 142:2017–2024. doi:10.3945/jn.112.164947.

Ma, Z. J., H. Misawa, and M. Yamaguchi. 2001. Stimulatory effect of zinc on insulin-like growth factor-I and transforming growth factor-beta1 production with bone growth of newborn rats. Int. J. Mol. Med. 8:623–628.

McCracken, B. A., M. E. Spurlock, M. A. Roos, F. A. Zuckermann, and H. R. Gaskins. 1999. Weaning anorexia may contribute to local inflammation in the piglet small intestine. J. Nutr. 129:613–619.

Moeser, A. J., C. V. Klok, K. A. Ryan, J. G. Wooten., D. Little, V. L. Cook, and A. T. Blikslager. 2007. Stress signaling pathways activated by weaning mediate intestinal dysfunction in the pig. Am. J. Physiol-Gastr. L. 292:G173-81.

NRC. 1998. Nutrient requirements of swine. 10th ed. Natl. Acad. Press, Washington, DC.

Peace, R. M., J. Campbell, J. Polo, J. Crenshaw, L. Russell, and A. Moeser. 2011. Spray-dried porcine plasma influences intestinal barrier function, inflammation, and diarrhea in weaned pigs. J. Nutr. 141:1312–1317. doi:10.3945/jn.110.136796.

Randhawa, P. K., K. Singh, N. Singh, and A. S. Jaggi. 2014. A review on chemical-induced inflammatory bowel disease models in rodents. Korean J. Physiol. Pharmacol. 18:279–288. doi:10.4196/kjpp.2014.18.4.279.

Roselli, M., A. Finamore, I. Garaguso, M. S. Britti, and E. Mengheri. 2003. Zinc oxide protects cultured enterocytes from the damage induced by *Escherichia coli*. J. Nutr. 133:4077–4082.

Secker, G. A., A. J. Shortt, E. Sampson, Q. P. Schwarz, G. S. Schultz, and J. T. Daniels. 2008. TGF-beta stimulated re-epithelialisation is regulated by CTGF and Ras/MEK/ERK signalling. Exp. Cell Res. 314:131–142. doi:10.1016/j.yexcr.2007.09.001.

Seo, H., Y. Cho, T. Kim, H. Shin, and I. Kwun. 2010. Zinc may increase bone formation through stimulating cell proliferation, alkaline phosphatase activity and collagen synthesis in osteoblastic MC3T3-E1 cells. Nutr. Res. Pract. 4:356–361. doi:10.4162/nrp.2010.4.5.356.

Shifflett, D. E., S. L. Jones, A. J. Moeser, and A. T. Blikslager. 2004. Mitogen-activated protein kinases regulate COX-2 and mucosal recovery in ischemic-injured porcine ileum. Am. J. Physiol. Gastrointest. Liver Physiol. 286:G906–913.

Smith, F., J. E. Clark, B. L. Overman, C. C. Tozel, J. H. Huang, J. E. Rivier, A. T. Blikslager, and A. J. Moeser. 2010. Early weaning stress impairs development of mucosal barrier function in the porcine intestine. Am. J. Physiol. Gastrointest. Liver Physiol. 298:G352–363.

Song, M., Y. Liu, J. A. Soares, T. M. Che, O. Osuna, C. W. Maddox, and J. E. Pettigrew. 2012. Dietary clays alleviate diarrhea of weaned pigs. J. Anim. Sci. 90:345–360. doi:10.2527/jas.2010-3662.

Song, Z. H., K. Xiao, Y. L. Ke, L. F. Jiao, and C. H. Hu. 2014. Zinc oxide influences mitogen-activated protein kinase and TGF-β1 signaling pathways, and enhances intestinal barrier integrity in weaned pigs. Innate Immun. doi:10.1177/1753425914536450.

Suer, S., D. Ampasala, M. F. Walsh, and M. D. Basson. 2009. Role of ERK/mTOR signaling in TGFbeta-modulated focal adhesion kinase mRNA stability and protein synthesis in cultured rat IEC-6 intestinal epithelial cells. Cell Tissue Res. 336:213–223. doi:10.1007/s00441-009-0776-z.

Wang, K., S. Ping, S. Huang, L. Hu, H. Xuan, C. Zhang, and F. Hu. 2013. Molecular mechanisms underlying the in vitro anti-inflammatory effects of a flavonoid-rich ethanol extract from Chinese propolis (poplar type). Evid. Based Complement. Alternat. Med. 2013:1–11.

Wang, X., D. Ou, J. Yin, G. Wu, and J. Wang. 2009. Proteomic analysis reveals altered expression of proteins related to glutathione metabolism and apoptosis in the small intestine of zinc oxide-supplemented piglets. Amino Acids 37:209–218. doi:10.1007/s00726-009-0242-y.

Xiao, K., Z. Song, L. Jiao, Y. Ke, and C. Hu. 2014. Developmental changes of TGF-β1 and smads signaling pathway in intestinal adaption of weaned pigs. PLoS ONE 9:E104589. doi:10.1371/journal.pone.0104589.

Yamaguchi, M., and M. Hashizume. 1994. Effect of beta-alanyl-L-histidinato zinc on protein components in osteoblastic MC3T3-El cells: Increase in osteocalcin, insulin-like growth factor-I and transforming growth factor-beta. Mol. Cell. Biochem. 136:163–169. doi:10.1007/BF00926077.

Zhang, X., Y. Zhao, Q. Chu, Z. Wang, H. Li, and Z. Chi. 2014. Zinc modulates high glucose-induced apoptosis by suppressing oxidative stress in renal tubular epithelial cells. Biol. Trace Elem. Res. 158:259–267. doi:10.1007/s12011-014-9922-x.

Zhu, L. H., J. X. Xu, S. W. Zhu, X. Cai, S. F. Yang, X. L. Chen, and Q. Guo. 2014. Gene expression profiling analysis reveals weaning-induced cell cycle arrest and apoptosis in the small intestine of pigs. J. Anim. Sci. 92:996–1006. doi:10.2527/jas.2013-7551.