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Article

Dietary Zn Deficiency Inhibits the Cell Proliferation Possibly via GPR39-Mediated Suppression of PI3K/AKT/mTOR Signaling Pathway in the Jejunum of Broilers

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Simple Summary: Zinc is an essential mineral element for broiler growth and development. In the present study, we found that dietary Zn deficiency decreased the amount of proliferating cell nuclear antigen positive cells and inhibited PI3K, AKT and mTOR phosphorylation along with down-regulation of GPR39 protein expression in the jujunum of broiler on d 42. These findings suggest that dietary Zn deficiency inhibit the cell proliferation possibly via GPR39-mediated suppression of PI3K/AKT/mTOR signaling pathway in the jejunum of broilers.

Abstract: A prior investigation revealed that a lack of Zinc (Zn) could hinder the intestinal cell proliferation in broiler chickens; however, the mechanisms responsible for this effect remain unclear. We aimed to investigate the possible mechanisms of dietary Zn deficiency in inhibiting the jejunal cell proliferation of broilers. For this study, a total of 112 chickens (21 d old) were randomly divided into two treatments (n = 7, 8 chickens per replicate cage): control (CON) and Zn deficiency groups. The duration of feeding was 21 d. Chickens in the CON group were provided with a basal diet containing an extra addition of 40 mg Zn/kg in the form of Zn sulfate, whereas chickens in the Zn deficiency group were given the basal diet with no Zn supplementation. The results indicated that, in comparison to the CON, Zn deficiency increased ($P < 0.05$) duodenal and jejunal crypt depth (CD) of broilers on d 28 and jejunal and ileal CD on d 35, and decreased ($P < 0.05$) duodenal, jejunal and ileal villus height/crypt depth (VH/CD) on d 28 and jejunal VH, jejunal and ileal villus surface area and VH/CD on d 35. Furthermore, Zn deficiency decreased ($P < 0.0001$) the amount of proliferating cell nuclear antigen positive cells, and down-regulated ($P < 0.01$) mRNA or protein expression levels of phosphatidylinositol 3-kinase (PI3K), phosphorylated PI3K, phosphorylated serine threonine kinase (AKT), phosphorylated mechanistic target of rapamycin (mTOR), G protein-coupled receptor 39 (GPR39), and extracellular regulated protein kinase, but up-regulated ($P < 0.05$) mRNA or protein expression levels of P38 mitogen activated protein kinase, c-jun N-terminal kinase (JNK) 1 and JNK2 and phosphorylated Protein Kinase C in the jejunum of broilers on d 42. It was concluded that dietary Zn deficiency inhibited the cell proliferation possibly via GPR39-mediated suppression of PI3K/AKT/mTOR signaling pathway in the jejunum of broilers.

Keywords: dietary Zn deficiency; broiler; Jejunum; cell proliferation; signaling pathway

1. Introduction

Microelement zinc (Zn), is an essential nutrient for broiler health [1]. Prior researches have shown that a lack of Zn may result in hindered growth performance [2], altered intestinal flora composition [3], impaired immune response [4], changed bone histomorphometry [5], and reduced meat quality in chickens [6]. In addition, dietary Zn deficiency was also reported to disturb the

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intestinal barrier of mice [7]. Cell proliferation is crucial for preserving the integrity and function of the small intestine [8]. Zhang and Guo (2009) reported that Zn could maintain normal barrier function of the small intestine mainly by promoting the proliferation of porcine small intestinal epithelial cells [9]. Therefore, Zn deficiency caused integrity disruption and intestinal barrier dysfunction by affecting cell proliferation. However, the molecular mechanism by which Zn deficiency inhibited cell proliferation in the small intestine is still unknown.

It is reported that intestinal cell proliferation can be regulated by phosphatidylinositol 3-kinase/protein kinase B/mechanistic target of rapamycin (PI3K/AKT/mTOR), protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) signaling pathways [10]. Previous studies indicated that Zn could promote proliferation of mouse C2C12 myoblasts, human gastric carcinoma cells or porcine intramuscular adipocytes through activating PI3K/AKT signaling pathway [11–13]. Kaltenberg et al. (2010) reported that Zn-accelerated mouse T cell proliferation depended on extracellular regulated protein kinase (ERK) activation [14]. Liang et al. (15) found that Zn could enhance the mouse osteogenetic capabilities by stimulating the proliferation of osteoblasts via the activation of PKC and MAPK signaling pathways. Furthermore, G protein-coupled receptor 39 (GPR39), a receptor that senses Zn^{2+}, plays a crucial role in controlling AKT, ERK, and PKC signaling pathways, and is responsible for the majority of Zn's biological effects. Therefore, GPR39 has the ability to detect alterations in extracellular Zn^{2+} levels and facilitate the transmission of Zn^{2+} signals [16]. In HT29 cells, GPR39 knockout attenuated Zn^{2+}-dependent AKT/mTOR and ERK1/2 activation [17]. In Caco2 cells, PKC expression was suppressed after silencing of GPR39 [18]. The aforementioned results imply that the suppression of cell proliferation caused by a lack of Zn might be associated with the GPR39-facilitated PI3K/AKT/mTOR, MAPK, or PKC signaling pathways. Furthermore, the jejunum has been shown to be the most sensitive in response to Zn deficiency among the small intestinal segments of broilers [19]. However, no related studies in broilers and other avian species have been reported before.

We hypothesized that dietary Zn deficiency would inhibit the jejunal cell proliferation of broilers possibly through the GPR39-mediated PI3K/AKT/mTOR, MAPK and PKC signaling pathways. Hence, in order to confirm the above hypothesis, this research was designed to determine the effect of dietary Zn deficiency on the histomorphology of the small intestine, cell proliferation, and mRNA and protein abundances of GPR39 and target genes involved in the aforementioned signaling pathways in the jejunum of broiler chickens.

2. Materials and Methods

The study's experimental protocols were carried out in compliance with the regulations set by the Animal Care Advisory Committee (Jiangsu, China) at Yangzhou University, with permit number SYXK (Su) 2021-0027.

2.1. Animals, Experiment Design and Treatments

One hundred and forty male broilers of the Arbor Acres (AA) breed (1-d-old) were acquired from Jiangsu Jinghai Poultry Group Co., Ltd. During 1-21 d, the broilers were provided with a complete diet consisting of corn and soybean meal. The diet was analyzed to contain 84.40 mg of Zn per kilogram and was formulated to meet or exceed the nutritional needs of starter broilers for all nutrients recommended by the National Research Council (NRC, 1994) and the Feeding Standard of Chicken in China (2004) [20,21] (Table 1). The Zn level in the diet was decided based on our previous study by Huang et al. (22). At 22 d of age, a total of 112 broilers were chosen based on their average body weight. These broilers were then divided into two treatment groups using a completely randomized design, with each group consisting of 7 replicate cages. Each replicate cage had 8 chickens. Birds were kept under a 24 h constant light schedule and given unrestricted access to experimental diets, and tap water containing an undetectable Zn. Broilers were fed a Zn-unsupplemented corn-soybean basal diet (Zn deficiency group) and the basal diet supplemented with 40 mg Zn/kg as Zn sulfate (reagent grade, Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) (the control group, CON) for 21 d. The supplemental level of 40 mg Zn/kg as Zn sulfate was

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designed based on the study of Liao et al. (23). The basal diet was formulated to meet or exceed the requirements for all nutrients except for Zn as recommended by NRC (1994) and the Feeding Standard of Chicken in China (2004) [20,21] (Table 1). The contents of Zn in the Zn deficiency group diet and the CON group diet were measured to be 22.23 and 64.36 mg/kg, respectively. Daily mortality was documented, bodyweight and the amount of feed consumed per replicate cage were measured on d 28, 35, and 42. These data were used to calculate the average daily feed intake (ADFI), average daily gain (ADG), feed to gain ratio (F/G), and mortality during d 22 to 42.

Table 1. Composition and nutrient contents of the broiler diets (as-fed basis).

Item	1 to 21 d of age	22 to 42 d of age	
		Control group	Zn deficiency group
Corn (%)	54.75	58.78	58.78
Soybean meal (%)	36.31	32.56	32.56
Soybean oil (%)	4.95	5.15	5.15
$CaHPO_4$ (%)[a]			
4	1.87	1.67	1.67
1)			
$CaCO_3$ (%)[a]	1.20	1.09	1.09
NaCl (%)[a]	0.30	0.30	0.30
DL-Methionine (%)[b]	0.31	0.15	0.15
Micronutrient (%)[c]	0.31	0.20	0.20
Corn starch[d]	0.00	0.10	0.10
Total (%)	100.00	100.00	100.00
Nutrient composition			
Metabolizable Energy (MJ/kg)[e]	12.75	12.98	12.98
Crude Protein (%)[f]	21.44	20.08	20.08
Lysine (%)[e]	1.10	1.01	1.01
Methionine (%)[e]	0.61	0.44	0.44
L-threonine (%)[e]	0.80	0.75	0.75
Tryptophan (%)[e]	0.24	0.22	0.22
Methionine + Cysteine (%)[e]	0.91	0.72	0.72
Ca (%)[f]	1.01	0.92	0.92
Non-phytate P (%)[e]	0.45	0.40	0.40
Zn (mg/kg)[f]	84.40	64.36	22.23

[a]Reagent grade. [b]Feed grade. [c]Provided per kilogram of diet for d 1 to 21: Vitamin A 12000 IU, Vitamin D_3 4500 IU, Vitamin E 33 IU, Vitamin K_3 3 mg, Vitamin B_1 (thiamin) 3 mg, Vitamin B_2 (riboflavin) 9.6 mg, Vitamin B_6 4.5 mg, Vitamin B_{12} 0.03 mg, Pantothenic acid calcium 15 mg, Niacin 54 mg; Folic acid 1.5 mg, Biotin 0.15 mg; Choline 700 mg, Cu ($CuSO_4 \cdot 5H_2O$) 6 mg, Fe ($FeSO_4 \cdot 7H_2O$) 40 mg, Zn ($ZnSO_4 \cdot 7H_2O$) 60mg, Mn ($MnSO_4 \cdot H_2O$) 110 mg, Se (Na_2SeO_3) 0.35 mg, I ($Ca(IO_3)_2 \cdot H_2O$) 0.35 mg; for d 22 to 42: Vitamin A 8000 IU, Vitamin D_3 3000 IU, Vitamin E 22 IU, Vitamin K_3 2 mg, Vitamin B_1 (thiamin) 2 mg, Vitamin B_2 (riboflavin) 6.4 mg, Vitamin B_6 3 mg, Vitamin B_{12} 0.02 mg, Pantothenic acid calcium 10 mg, Niacin 36 mg; Folic acid 1.0 mg, Biotin 0.10 mg; Choline 500 mg, Cu ($CuSO_4 \cdot 5H_2O$) 6 mg, Fe ($FeSO_4 \cdot 7H_2O$) 30 mg, Mn ($MnSO_4 \cdot H_2O$) 80 mg, Se (Na_2SeO_3) 0.35 mg, I ($Ca(IO_3)_2 \cdot H_2O$) 0.35 mg. [d]$ZnSO_4 \cdot 7H_2O$ added in place of the equivalent weight of corn starch to produce the control group diet. [e]Calculated values. [f]Values determined by analysis, and each value is based on triplicate determinations.

2.2. Sample Collections and Preparations

Samples of the diets were gathered and examined for contents of calcium, Zn, and crude protein. The tap water was collected for analysis of Zn content. At 28, 35 and 42 d of age, chickens were subjected to overnight fasting, but water was available ad libitum. At the end of the fasting period, 1 chicken of the body weight close to the cage average body weight was selected from each replicate cage at 28 and 35 d of age, respectively, while 3 chickens of the body weight close to the cage average body weight were selected from each replicate cage at 42 of age, and then, the selected chickens were

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anesthetized by intraperitoneal injection of propofol (20 mg/kg body weight), and killed by cervical dislocation. At 28 or 35 d of age, the middle 2 cm of the duodenum, jejunum and ileum of the chickens were cut and fixed in 4% paraformaldehyde to analyze the small intestine histological morphology. At 42 d of age, the middle 2 cm of the jejunum of 1 of the killed 3 chickens was cut and fixed in 4% paraformaldehyde to analyze the number of proliferating cell nuclear antigens (PCNA), and then the mucosa of the remaining part of the jejunum and the jejunum of the remaining two chickens were scraped, packed into 1.5 mL centrifuge tubes, snap-frozen with liquid nitrogen, and placed in a -80°C for analyses of mRNA and protein expression levels.

2.3. Analyses of Dietary Crude Protein, Calcium and Zn Contents, Zn Concentration in Tap Water, and the Jejunal PCNA Positive Cells

We employed the kjeldahl method to determine the crude protein contents in diets [24]. To determine calcium and Zn contents in the diets and Zn concentration in tap water, the 5110 ICP-OES (Agilent Technologies Australia (M) Pty Ltd, Australia), a plasma optical emission spectrometer was employed. The jejunal specimens were subjected to H&E staining followed by their visualization under an Olympus CKX53 light microscope (Tokyo, Japan). The number of PCNA positive cells in the jejunum was analyzed by immunohistochemistry. In short, the slides underwent a treatment with an antigen repair solution for a period of 10 min. After being exposed to 3% hydrogen peroxide, the slides were blocked with goat serum for a duration of 30 min. Subsequently, the slides were placed in a humid chamber at 4 °C and incubated overnight with the primary antibody, which had been diluted. On the following day, the slides were then treated with a secondary antibody and incubated for 2 h at room temperature. Afterward, the slides underwent staining with 3,3'-diaminobenzidine (DAB) and hematoxylin. At the end, the slides were affixed with a neutral resin glue, overlaid with coverslips, and observed under an Olympus CKX53 light microscope (Tokyo, Japan) at a 100× magnification.

2.4. RT-qPCR

Total RNA from the jejunum was isolated using the Vazyme RNA extraction kit. SYBR Green RT-qPCR was used to determine the mRNA expression levels in the jejunum, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin serving as reference genes [25]. The expression level of each target gene's mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method [26]. All primer sequences are listed in Table 2.

Table 2. Primer sequences for real-time PCR amplification*.

Genes	GenBank ID	Primer sequences	Product length (bp)
PI3K	XM_046923916.1	F: 5'-CTTCTGGAGTCCTATTGTCG-3' R: 5'- CACCTTCTGGGTCTCATCTT-3'	132
AKT	NM_205055	F: 5'-GCCGTGAGCCCAGTTAGG-3' R: 5'-AGCTACTTATGGCTGCGGGA-3'	153
mTOR	XM_417614.6	F: 5'-AACCACTGCTCGCCACAATGC-3' R: 5'-CATAGGATCGCCACACGGATTAGC-3'	120
P38 MAPK	XM_040691290.1	F: 5'-ACGTGCAGTTCCTCATATACCA-3' R: 5'-TGTCGAGCCAAGCCAAAATC-3'	145
ERK	NM_204150.1	F: 5'-TCTTACTGCGCTTCAGGCAT-3' R: 5'-AATGTGGTCGTTGCTGAGGT-3'	158
JNK1	XM_040675398.1	F:5'-GCTGGTTATAGACGCCTCGA-3' R: 5'-GCTCCCTCTCATCTAACTGCT-3'	137
JNK2	NM_001396829.1	F: 5'-AGAATCAAACCCACGCAAAA-3' R: 5'-ATCAGTTCCATAACCAAATA-3'	148
PKC	NM_001012804.2	F: 5'-GGCGGACAGGAAGAATACAGAGG-3'	146

		R: 5'-GAAGCTGTGTCAGGAATGGTGGTT-3'	
GPR39	NM_001080105.1	F: 5'-GCTGTAAAGATTGGTAAGCACTGA-3' R: 5'-ATATGCACAAGTCTGAGCGGT-3'	151
β-actin	NM_205518.1	F: 5'- CAGCCATCTTTCTTGGGTAT-3' R: 5'- CTGTGATCTCCTTCTGCATCC-3'	169
GAPDH	NM_204305.1	F: 5'-CTTTGGCATTGTGGAGGGTC-3' R: 5'-ACGCTGGGATGATGTTCTGG-3'	128

*PI3K, phosphatidylinositol 3-kinase; *AKT*, serine threonine kinase; m*TOR*, target of rapamycin; *P38MAPK*, P38 mitogen activated protein kinase; *ERK*, extracellular regulated protein kinase; *JNK*, C-jun N-terminal kinase; *PKC*, protein kinase C; GPR39, G protein-coupled receptor 39. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; F, forward; R, reverse.

2.5. Western Blot

The Western blot technique was carried out based on a previous study conducted by Hu et al. (27). Total Protein Extraction Kit (Beyotime, China) was used for the extraction of jejunal total proteins. To determine the proteins concentration, a BCA protein detection kit (ThermoFisher, USA) was employed. Each sample was diluted to a final concentration of 4 ug/uL using RIPA lysis buffer. SDS-PAGE was employed to transfer the proteins onto the NC membrane, which was then blocked with 5% skim milk for 2 h at room temperature and exposed to the primary antibody overnight at 4 °C. Subsequently, the NC membrane was rinsed with TBST, followed by a 2-h incubation with the secondary antibody. The images were captured using the AllDoc x system (Tanon). The quantification of proteins was conducted using β-actin as the internal reference.

2.6. Statistical Analyses

Statistical analyses were performed using SAS software (version 9.4, 2013). The student's t test was used to identify significant differences between the two treatment groups, with statistical significance being defined as $P \leq 0.05$.

3. Results

3.1. Growth Performance

In our previous study [28], it was observed that dietary Zn deficiency had no impact ($P > 0.05$) on ADG, ADFI, and mortality of broilers compared to the CON. However, it did lead to an increase ($P < 0.05$) in F/G of broilers from d 22 to 42.

3.2. Intestinal Histological Morphology

As shown in Table 3, compared with the CON, Zn deficiency increased ($P < 0.05$) duodenal and jejunal crypt depth (CD), but decreased ($P < 0.05$) duodenal, jejunal and ileal villus height/crypt depth (VH/CD) of broilers on d 28. However, Zn deficiency did not affect ($P > 0.05$) the duodenal, jejunal and ileal villus height (VH) and villus surface area (VSA), and ileal CD of broilers on d 28.

Table 3. Effect of dietary Zn deficiency on histological morphology of the small intestine in broilers at 28 d of age.

Groups		CON group[1]	Zn deficiency group[1]	SEM	*P*-value
Duodenum	Villus height (μm)	2071	1939	54.8	0.177
	Crypt depth (μm)	199[b]	218[a]	3.10	0.003
	Villus height/crypt depth	10.35[a]	8.96[b]	0.261	0.008
	Villus surface area (mm^2)	1.299	1.264	0.031	0.555
Jejunum	Villus height (μm)	1736	1577	96.4	0.282
	Crypt depth (μm)	186[b]	208[a]	11.89	0.031

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	Villus height/crypt depth	9.65[a]	7.73[b]	0.207	0.032
	Villus surface area (mm²)	1.030	0.818	0.072	0.066
Ileum	Villus height (μm)	910	871	18.9	0.324
	Crypt depth (μm)	185	213	10.19	0.084
	Villus height/crypt depth	5.16[a]	4.29[b]	0.222	0.020
	Villus surface area (mm²)	0.405	0.360	0.016	0.254

[1]Values are the means of 5-7 replicate cages of 1 bird per replicate cage (n = 5-7). [a, b]Means with different superscripts within the same column differ ($P < 0.05$).

As shown in Table 4, compared with the CON, Zn deficiency increased ($P < 0.05$) jejunal and ileal CD, and decreased ($P < 0.05$) jejunal VH, jejunal and ileal VH/CD and VSA of broilers on d 35. However, Zn deficiency did not affect ($P > 0.05$) the duodenal and ileal VH and VSA, and ileal CD of broilers on d 35. In addition, Zn deficiency increased ($P < 0.001$) duodenal and jejunal CD, and decreased ($P < 0.05$) duodenal, jejunal and ileal VH, jejunal VH and VSA of broilers on d 42 as described in our previous study [28].

Table 4. Effect of dietary Zn deficiency on histological morphology of the small intestine in broilers at 35 d of age.

Groups		CON group[1]	Zn deficiency group[1]	SEM	*P*-value
Duodenum	Villus height (μm)	1612	1546	26.0	0.133
	Crypt depth (μm)	169	166	2.54	0.534
	Villus height/crypt depth	9.70	9.30	0.170	0.282
	Villus surface area (mm²)	1.135	1.113	0.032	0.734
Jejunum	Villus height (μm)	1521[a]	1303[b]	43.0	0.029
	Crypt depth (μm)	168[b]	180[a]	0.703	0.050
	Villus height/crypt depth	8.73[a]	7.39[b]	0.276	0.009
	Villus surface area (mm²)	0.936[a]	0.731[b]	0.026	0.009
Ileum	Villus height (μm)	819	770	13.7	0.241
	Crypt depth (μm)	158[b]	175[a]	0.644	0.001
	Villus height/crypt depth	5.21[a]	4.43[b]	0.080	0.002
	Villus surface area (mm²)	0.504[a]	0.400[b]	0.008	0.003

[1]Values are the means of 6-7 replicate cages of 1 bird per replicate cage (n = 6-7). [a, b]Means with different superscripts within the same column differ ($P \leq 0.05$).

3.3. The Amount of PCNA Positive Cells

As shown in Table 5, compared with the CON, Zn deficiency decreased ($P < 0.0001$) the amount of PCNA positive cells in the jejunum of broilers on d 42.

Table 5. Effect of dietary Zn deficiency on the amount of PCNA[2] positive cells in the jejunum of broilers at 42 d of age.

Groups	PCNA positive cells (AOD value)[3]
CON group[1]	0.3591[a]
Zn deficiency group[1]	0.1235[b]
SEM	0.0034
P-value	< 0.0001

[1]Values are the means of 7 replicate cages of 1 bird per replicate cage (n = 7). [2]PCNA: Proliferating Cell Nuclear Antigen. [3]The amount of PCNA positive cells: the average optical density (AOD) value was used to reflect the amount of PCNA positive cells, and the higher the AOD value, the higher the amount of PCNA positive cells. The calculation formula of the AOD value was: AOD = IOD/area, where IOD stands for integrated optical density that was the total responsive strength of all PCNA positive cells, and area was one where all PCNA positive cells

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are located within the whole selected photographed field. Five fields per chamber were photographed under a light microscope for the quantification (100 ×). [a, b]Means with different superscripts within the same column differ ($P < 0.05$).

3.4. mRNA Expression Levels

As shown in Table 6, compared with the CON, Zn deficiency down-regulated ($P < 0.01$) mRNA expression levels of *PI3K*, *ERK* and *GPR39*, and up-regulated ($P < 0.05$) mRNA expression levels of P38 mitogen activated protein kinase (*P38 MAPK*), c-jun N-terminal kinase (*JNK*) 1 and *JNK2* in the jejunum of broilers on d 42. However, Zn deficiency did not affect ($P > 0.05$) mRNA expression levels of *AKT*, *mTOR* and *PKC* of broilers on d 42.

Table 6. Effect of dietary Zn deficiency on mRNA expression levels of target genes involved in the cell proliferation-related signaling pathways in the jejunum of broilers at 42 d of age.

Groups		CON group[1]	Zn deficiency group[1]	SEM	*P*-value
PI3K[3] mRNA	RQ[2]	1.209[a]	0.970[b]	0.0658	< 0.0001
AKT[3] mRNA		1.011	1.016	0.0581	0.9598
mTOR[3] mRNA		0.997	1.009	0.0492	0.8739
P38 MAPK[3] mRNA		0.621[b]	1.003[a]	0.0375	< 0.0001
JNK1[3] mRNA		0.826[b]	1.006[a]	0.0454	0.0254
JNK2[3] mRNA		0.728[b]	1.004[a]	0.0300	0.0002
ERK[3] mRNA		1.210[a]	1.004[b]	0.0358	0.0018
PKC[3] mRNA		1.020	1.002	0.0264	0.7378
GPR39[3] mRNA		1.669[a]	1.004[b]	0.0360	< 0.0001

[1]Values are the means of 5-7 replicate cages of 3 birds per replicate cage (n = 5-7). [2]Values of mRNA abundance levels of target genes were calculated as the relative quantities (RQ) of *PI3K*、*AKT*、m*TOR*、*P38 MAPK*、*JNK1*、*JNK2*、*ERK*、*PKC* or *GPR39* mRNA to the geometric mean of internal reference genes *β-actin* and *GAPDH* mRNA using $2^{-\Delta\Delta Ct}$. [3]*PI3K*, phosphatidylinositol 3-kinases; *AKT*, protein-serine-threonine kinase; *JNK*, C-jun N-terminal kinase; *P38 MAPK*, mitogen activated protein kinase; m*TOR*, mechanistic target of rapamycin; *ERK*, extracellular regulated protein kinase; *PKC*, protein kinase C; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. [a, b]Means with different superscripts within the same column differ ($P < 0.05$).

3.5. Protein Expression Levels

As shown in Table 7, compared with the CON, Zn deficiency decreased ($P < 0.05$) protein abundances of phosphorylated PI3K (p-PI3K), p-AKT, p-mTOR and GPR39 in the jejunum of broilers on d 42. However, Zn deficiency did not affect ($P > 0.05$) protein abundances of PI3K, AKT, mTOR, P38 MAPK, p-P38 MAPK, JNK, p-JNK, ERK, p-ERK and PKC.

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Table 7. Effect of dietary Zn deficiency on protein expression levels of target proteins or phosphorylated proteins involved in the cell proliferation-related signaling pathways in the jejunum of broilers at 42 d of age.

Groups		CON group[1]	Zn deficiency group[1]	SEM	*P*-value
PI3K[3]	RQ[2]	0.59	0.54	0.0111	0.0717
p-PI3K[3]		0.48[a]	0.29[b]	0.0102	0.0012
AKT		0.81	0.86	0.0097	0.1414
p-AKT		0.62[a]	0.43[b]	0.0109	0.0008
mTOR[3]		0.58	0.54	0.0203	0.2690
p-mTOR		0.33[a]	0.29[b]	0.0084	0.0213
P38 MAPK[3]		0.64	0.65	0.0058	0.7956
p-P38 MAPK		0.48	0.48	0.0129	0.8395
JNK[3]		0.67	0.63	0.0152	0.1793
p-JNK		0.24	0.22	0.0204	0.6197
ERK[3]		0.57	0.58	0.0115	0.9842
p-ERK		0.51	0.46	0.0180	0.2416
PKC[3]		0.52	0.52	0.0068	0.7377
p-PKC		0.30[b]	0.33[a]	0.0063	0.0142
GPR39[3]		0.84[a]	0.74[b]	0.0211	0.0081

[1]Values are the means of 5-7 replicate cages of 3 birds per replicate cage (n = 5-7). [2]Values of target protein expression levels were expressed by the relative quantities (RQ) of target protein band pixel density to the internal reference band pixel density of β-actin. [3]PI3K, phosphatidylinositol 3-kinase; p-PI3K, phosphorylated phosphatidylinositol 3-kinase; AKT, serine threonine kinase; p-AKT, phosphorylated serine threonine kinase; mTOR, mechanistic target of rapamycin; p-mTOR, phosphorylated mechanistic target of rapamycin; P38 MAPK, P38 mitogen activated protein kinase; p-P38 MAPK, phosphorylated P38 mitogen activated protein kinase; ERK, extracellular regulated protein kinase; p-ERK, phosphorylated extracellular regulated protein kinase; JNK, C-jun N-terminal kinase; p-JNK, phosphorylated C-jun N-terminal kinase; PKC, protein kinase C; p-PKC, phosphorylated protein kinase C; GPR39, G protein-coupled receptor 39. [a, b]Means with different superscripts within the same column differ ($P < 0.05$).

4. Discussion

In the present study, we revealed that dietary Zn deficiency disrupted the small intestinal structure, and decreased the amount of PCNA positive cells, mRNA or protein expression levels of PI3K, ERK, GPR39, p-PI3K, p-AKT, p-mTOR, but increased mRNA or protein expression levels of P38 MAPK and p-PKC in the jejunum of broilers on d 42. These results indicate that dietary Zn deficiency inhibited the jejunal cell proliferation of broilers possibly through the GPR39- mediated PI3K/AKT/mTOR signaling pathway, thereby impairing the small intestinal integrity and barrier function, which has supported our hypothesis. These findings have been not reported before, and provided new insights into dietary rational Zn addition in the broiler production in order to improve the intestinal health of broilers.

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The small intestine is a vital organ in the digestive system, where most nutrient absorptions take place. Previous studies have demonstrated that Zn deficiency had a negative impact on the villi structure of the small intestine of broilers [1,28]. The destruction of the intestinal physical barrier can cause nutrient absorption deficiency and thus affect the growth performance of animals. Intestinal morphology indexes, such as VH, VSA, CD, and VH/CD ratio are commonly used indices to assess intestinal development and barrier function [29]. A previous study in our laboratory indicated that Zn deficiency decreased jejunal VH and VSA, and increased duodenal and jejunal CD, but decreased duodenal, jejunal and ileal VH/CD ratios of broiler on d 42 [28]. In the present research, we found that Zn deficiency increased duodenal and jejunal CD, and decreased duodenal, jejunal and ileal VH/CD ratios of broiler on d 28, while Zn deficiency increased ileal CD, and decreased jejunal VH, jejunal and ileal VSA and VH/CD of broiler on d 35. The above results indicate that dietary Zn deficiency impaired the small intestinal histomorphology, which in turn damaged the small intestinal integrity and barrier function.

Wu et al. (28) demonstrated that the damage of Zn deficiency on the jejunum of broilers was more obvious. Moreover, in our present study, dietary Zn deficiency caused more serious damage to the jejunum of broilers. Therefore, the jejunum of broilers on d 42 was used for the follow-up examinations. Cell proliferation plays an important role in maintaining the intestinal integrity and barrier function [7]. The PCNA is considered to be a marker of cell proliferation in various tissues [30]. Shao et al. (31) and Kang et al. (32) reported that Zn improved the integrity and barrier function via increasing the amount of PCNA positive cells in Caco-2 cells or mice. Similar results were observed in the current study that Zn deficiency reduced the number of PCNA positive cells in the jejunum of broilers, suggesting that Zn deficiency would impair the intestinal integrity and barrier function by inhibiting cell proliferation.

Previous studies have established the vital role of PI3K/Akt/mTOR and PKC signaling cascade in cellular growth and proliferation [33,34]. Dai et al. (35) reported that dieckol inhibited cell proliferation through impeding the expression of PI3K, AKT and mTOR. Ohashi et al. (11) found that Zn accelerated proliferation of C2C12 myogenic cells through boosting phosphorylation of PI3K/Akt. Liang et al. (15) demonstrated that Zn could promote osteoblast proliferation by activating PKC signaling pathway. Similar results were found in the current study that dietary Zn deficiency decreased the cell proliferation associated with down-regulation of p-PI3K, p-AKT and p-mTOR protein expressions. However, the protein expression of p-PKC in the jejunum of broilers was increased by Zn deficiency, which is different from a previous study in 3T3 cells [36], which showed a down-regulated p-PKC protein expression. This discrepancy might be attributed to different study models, the way or the dose of supplemental Zn.

The GPR39 is a Zn-sensing receptor whose activation leads to activation of PI3K/Akt/mTOR signaling cascade in mammals [37,38]. In normal immortalized human hepatocyte LO2 cells and HCC cell lines, silencing GPR39 reduced protein expressions of p-PI3K, p-AKT and p-mTOR [38]. In the present study, we found that Zn deficiency inhibited PI3K, AKT and mTOR phosphorylation along with down-regulation of GPR39 protein expression. The above findings suggest that Zn deficiency might inhibit the PI3K/AKT/mTOR signaling pathway via restraining GPR39 expression.

5. In Conclusions

Dietary Zn deficiency inhibits the cell proliferation possibly via GPR39-mediated suppression of PI3K/AKT/mTOR signaling pathway in the jejunum of broilers. Further studies need be carried out using the primary cultured jejunal epithelial cells as well as gene over-expression and RNA silencing tools to address and confirm the above possible mechanisms.

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