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Research Article

The Effects of Zinc Methionine Chelate and $ZnSO_4$ on the Growth Performance and Immune Function of the Weaned Piglets and on IPEC-J2 Cell Immune Function

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Abstract

Zinc Methionine chelate (Met-Zn) shows a better palatability, stability, and bioactivity than traditional zinc preparations, therefore this study evaluated the effect on the growth performance and immunologic functions of the weaned piglets. Two *in vivo* tests were conducted: (I) cross-breeding piglets [duroc × (landrace × large white pigs)] were fed 80 mg/kg $ZnSO_4$ or 20, 40, 60, or 80 mg/kg Met-Zn after the weaning on day 21.The content of serum globulin and lymphocyte transformation rate were measured on days 21, 35, 45 and 60; (II) another group of piglets weaned on day 28 days were fed 80 mg/kg $ZnSO_4$ or 80 mg/kg Met-Zn after orally administrated of *Escherichia coli.* The levels of some immune factors in the small intestine were measured after the feeding for one month. An *in vitro* experiment studied the expression of some immune factors and zinc transporters in the porcine small intestinal epithelial cells (IPEC-J2) after treatments with $ZnSO_4$+LPS and Met-Zn+LPS. Both Met-Zn and $ZnSO_4$ increased the lymphocyte transformation rate and the content of serum globulin. But, Met-Zn showed better effect than ZnSO4 in improving the growth performance, particularly the average daily gain, after *E. coli* insults. With *E. coli* insults, Met-Zn promoted the expression of TNF-α and IL-6 in the anterior and middle segments of the small intestine respectively, but inhibited the expression of IL-8 in the middle segment. $ZnSO_4$ promoted the expression of IL-6 in the posterior segment of the small intestine, but inhibited the expression of TNF-α in the middle segment. Both Met-Zn and $ZnSO_4$ dose-dependently increased the expression levels of TNF-α, IL-6, and IL-8 in IPEC-J2 cells after the LPS stimulation. In summary, Met-Zn improved the growth performance of piglets and changed the immunologic functions.

Keywords: *Met-Zn, Piglets, Growth performance, Intestinal tract, Immunity*

Çinko Metionin Şalat ve $ZnSO_4$'ın Sütten Kesilmiş Domuz Yavrularında Büyüme Performansı ve Bağışıklık İle IPEC-J2 Hücre İmmun Fonksiyonları Üzerine Etkileri

Öz

Çinko metionin şalatı geleneksel çinko preprasyonları ile karşılaştırıldığında daha lezzetli, stabil ve bioaktiftir. Bu çalışmada sütten kesilmiş domuz yavrularında çinko metionin şalatın büyüme performansı ve immunolojik fonksiyonlar üzerine etkisi çalışılmıştır. İki *in vivo* test uygulanmıştır: (I) Melez domuz yavruları [Duroc x (Landrace x Büyük Beyaz domuz)] 80 mg/kg $ZnSO_4$, veya 20, 40, 60 ve 80 mg/kg Met-Zn ile 21 gün süresince sütten kesme sonrasında beslendi. Serum globülin miktarı ve lenfosit transformasyon oranı 21, 35, 45 ve 60. günlerde ölçüldü. (II) 28. Günde sütten kesilmiş olan ve ağız yoluyla *Escherichia coli* uygulanan domuz yavruları 80 mg/kg $ZnSO_4$ veya 80 mg/kg Met-Zn ile beslendi. Bir aylık besleme sonrasında, ince barsaklarda bazı immun faktörlerin seviyeleri ölçüldü. $ZnSO_4$+LPS ve Met-Zn+LPS uygulaması sonrasında domuz ince barsak epitel hücrelerinde (IPEC-J2) bazı immun faktörler ve çinko transporterlerinin ekspresyonu *in vitro* olarak araştırıldı. Hem Met-Zn hem de $ZnSO_4$ lenfosit transformasyon oranı ve serum globülin miktarını artırdı. Met-Zn büyüme performansını iyileştirmede, özellikle de *E. coli* maruziyeti sonrasında ortalama günlük kilo kazanımınd $ZnSO_4$'tan daha iyi etki gösterdi. *E. coli* maruziyetinde, Met-Zn ince barsakların anterior ve orta bölümde sırasıyla TNF-α ve IL-6 ekspresyonlarını uyarırken orta bölümde IL-8 ekspresyonunu inhibe etti. $ZnSO_4$ ince barsakların posterior bölümünde IL-6 ekspresyonlarını uyarırken orta bölümünde TNF-α ekspresyonunu inhibe etti. Hem Met-Zn hem de ZnSO4 LPS stimulasyonu sonrasında IPEC-J2 hücrelerinde TNF-α, IL-6 ve IL-8 ekspresyon seviyelerinde doza bağlı artmeya neden oldu. Özet olarak, Met-Zn domuz yavrularında büyüme performansında iyileşmeye ve immunolojik fonksiyonlarda değişime neden oldu.

Anahtar sözcükler: *Met-Zn, Domuz yavrusu, Büyüme performansı, İntestinal kanal, İmmunite*

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INTRODUCTION

Zinc is an essential microelement in organisms, which adjusts multiple physiological functions, especially the immunological functions. Zinc maintains the development of immune organs and modulates cellular immunity and humoral immunity functions. Lack of zinc hinders the development of thymus gland and spleen of animals, and even results in the atrophy [1,2]. Zinc also has an influence on the number, transformation rate, phagocytosis and secretion function of immune cells. Lack of zinc significantly affects the number and functions of these cells, and reduces cell-mediated immune response, therefore influences the immune function of animals [3]. In the animal production industry, zinc can be added to diet in three manners, that is, inorganic zinc, simple organic zinc and zinc amino acid chelate. Although inorganic zinc is cheap, it shows a series of problems in the actual production. For example, the absorption and utilization efficiency of inorganic zinc is generally low, leading to the excessive supplementation and the environmental pollution resulted from unabsorbed zinc [4]. Besides, it is easy for inorganic zinc to influence the absorption and utilization of other nutritional factors [5]. In contrast, zinc amino acid chelates, such as Zinc Methionine chelate (Met-Zn) and Zinc glycine chelate, show many advantages. First, the palatability of inorganic zinc, similar to other inorganic salt, is poor due to the metallic taste. However, the smell of zinc amino acid chelates is close to the amino acid, and its palatability is better to promote animals to eat [6-8]. Moreover, zinc ion interacts with amino acid to form a five-membered (or six-membered) ring through the strong coordination bond in zinc amino acid chelates. Therefore, zinc ions are completely bound to the chelate ring, and the internal charge of zinc amino acid chelates tends to be neutral, resulting in a very table chemical structure. However, inorganic zinc and simple organic zinc show a poor stability because only simple ionic bond is formed in these chemical compounds, thus they easily interact with other nutrient substances [9,10]. The stable chemical structure of Met-Zn prevents Met-Zn from the combination with anti-nutritional factors, such as phytic acids forming insoluble compounds, and from the impairment of gastric acid. Consequently, metal trace elements smoothly enter the absorption site, which guarantees a high-efficient absorption [11]. Met-Zn and other trace element amino acid chelates generally show higher biological effects than the trace element sand amino acids, as well as exert many special physiological actions, such as improving the utilization rate of protein and vitamin, participating in intracellular redox reactions and modulating enzyme activities in organisms [8]. These effects are considered to improve the activity of immune cells and immune response, whereby enhancing the effects of cellular and humoral immunity of animals [12]. At present, early weaning of piglets is widely adopted to increase production efficiency. Early weaning influences the immune system of piglets in several aspects, especially hindering piglets to obtain maternal immune factors, which reduces the levels of antibodies in piglets. Maternal passive immunity plays a critical role in immune response of piglets, before the immune function of piglets gradually develop after the 4-5 weeks old. Therefore, the earlier the weaning time, the greater influence on the immunity. Early weaning also causes some stress reactions in piglets, resulting in the immunosuppression [6]. The intestinal tract is the largest digestive and absorption organ and functions as a protective screen against foreign harmful bacteria. However, the intestinal absorption and immunity are interfered by a series of physical, and environmental changes after the early weaning, which reduce the nutrient absorbing ability, makes the intestinal tract easy to be infected by pathogenic bacteria, and finally influence the growth of piglets [13].

The present study aimed to compare the effects of Met-Zn and zinc sulfate ($ZnSO_4$) on improving the growth performance and immune functions of early weaned piglets. The *in vivo* test initially evaluated the effect of Met-Zn at different dosages on the content of serum globulin and lymphocyte transformation rate of the early weaned piglets. Then the effects of Met-Zn and $ZnSO_4$ on the growth performance and immune functions were investigated in the early weaned piglets that were subjected to *Escherichia coli*. The *in vitro* test was performed in the porcine small intestinal epithelial cells (IPEC-J2) after the stimulation of LPS. Met-Zn and $ZnSO_4$ were added to the cells to evaluate their influence on the expression of immune factors and zinc transporters.

MATERIAL and METHODS

Ethics Statement

All animal work was approved by the University of Hunan Agricultural Animal Care Committee (Changsha, Hunan Province, China; Permit Number: 27-2956; Date: 2017. 6. 5). The experimental procedures were conducted in accordance with the Chinese guidelines for animal welfare.

The In Vivo Test

Two *in vivo* tests were conducted. In the first, a total of 288 duroc × (landrace × large white pig, DLY) three-way cross-breeding piglets weaned at the age of 21 days were divided into six groups: control group (basal diet group), $ZnSO_4$ group (adding 80 mg/kg $ZnSO_4$ to the diet) and four Met-Zn groups (respectively adding 20, 40, 60, and 80 mg/kg Met-Zn to the diet). Met-Zn and $ZnSO_4$ were purchased from Xingjia Bio-Engineering Co., Ltd. Each group included 8 repetitions and each repetition included 6 piglets. The test was carried out in two periods: period one: 21 - 35 days old; period two: 35 - 60 days old. The selected piglets were fed with different basal diets in the two periods *(Table 1)*, and the nutritional level was designed according to the

Table 1. *Composition and nutrient levels of basal diets*

Ingredients	The First Stage (%)	The Second Stage (%)
Corn	54.00	60.00
Dehulled soybean meal	8.00	12.00
Extruded Soybean	8.00	8.00
Whey powder	5.00	0.00
Fish meal	0.00	2.50
Fermented soybean meal	10.00	6.00
Glucose	2.50	2.50
Plasma protein	3.50	0.00
Soybean protein concentrate	0.00	2.00
Soybean oil	2.00	2.00
$CaHPO_4$	0.70	0.70
Limestone	0.70	0.70
Citric acid	1.30	1.30
L-Lyso•HCl	0.30	0.30
Premix[1]	4.00	2.00
Nutrient Levels[2]		
DE (MJ/kg)	14.65	14.48
CP (%)	20.5	20.00
TP (%)	0.60	0.60
Ca (%)	0.70	0.70
Lys (%)	1.45	1.30
Met (%)	0.48	0.44
Thr (%)	0.95	0.84
Try (%)	0.29	0.26
Zn (mg/kg)	22	22

[1] The full price of feed per kilogram of premix provided: Vit. A, 1500 IU; Vit. D_3, 200 IU; Vit. E, 85 IU; D-pantothenic acid, 35 mg; Vit. B_2, 12 mg; Folic acid, 1.5 mg; Nicotinic acid, 35 mg; Vit. B_1, 3.5 mg; Vit. B_6, 2.5 mg; Biotin, 0.2 mg; Vit. B_{12}, 0.05 mg; Cu (as copper sulfate), 15 mg; Fe (as ferrous sulfate), 100 mg; Mn, 20 mg; I (as calcium iodate), 1 mg; Se (as sodium selenite), 0.35 mg; Co (as cobalt sulfate) 0.2 mg; Cr (as chromium picolinate), 0.2 mg
[2] DE, CP, and TP are measured values. Other nutrient levels are calculated value

standard of NRC (2012) [14]. In the second, a total of 144 DLY three-way cross-breeding piglets weaned at the age of 28 days were administrated orally with 3×10^9 colony-forming units of *Escherichia coli* (O157: H7 strain) to induce immunological stress. *Escherichia coli* (O157: H7) strain was gifted by Prof. Tan (Key Laboratory of Agro-Ecological Processes in Subtropical Region, Hunan Research Center of Livestock & Poultry Sciences, South-Central Experimental Station of Animal Nutrition and Feed Science in Ministry of Agriculture, Institute of Subtropical Agriculture, the Chinese Academy of Sciences, China). Then the piglets were divided into three treatments: control group (basal diet group); $ZnSO_4$ group (basal diet + 80 mg/kg $ZnSO_4$); Met-Zn group (basal diet + 80 mg/kg Met-Zn). Each treatment included 8 repetitions, and each repetition included 6 piglets. The preliminary trial period was 3 days, and formal trial period was 30 days (that is, at the age of 28~58 days). The level of basal diet referred to the nutrient level and composition of diet in the second period in *Table 1*. Body weight (BW) of pigs was measured at the beginning and end of the experiment, and the feed consumption of each group was monitored every day during the experiment period.

Sample Collection and Tests

In the first part of the test, a male and a female piglet were randomly selected in each repetition. 5 mL blood was collected from the precaval vein after 12 h of fasting at the age of 21, 35, 45, and 60 days. Lymphocyte was isolated from the blood to evaluate the transformation rate of peripheral blood lymphocyte. The transformation rate of peripheral blood lymphocyte was measured with methyl thiazolyl tetrazolium (MTT) colorimetric method [15]. The serum was obtained by the centrifuge and used to inspect the serum globulin content. To test the serum globulins, the contents of total protein and albumin were measured. The contents of total protein minus that of albumin content was globulin content. The total protein was measured with the biuret method, and albumin was measured with Bromcresol green dye method [16]. In the second part of the test, a male and a female piglet were selected and euthanized in each repetition. The small intestine was cleaned with physiological saline and cut to three segments: the anterior segment included the duodenum and jejunum; the middle segment included the anterior and middle part of ileum; the posterior segment included the posterior part of ileum. Each segment of the small intestine was sampled and frozen in liquid nitrogen for long-term preservation.

Enzyme-Linked Immunosorbent Assay (ELISA)

The C3 and C4 complements in the small intestine were determined by the commercially available ELISA kits (Sigma, St. Louis, MO, USA) following the manufacture protocols. Briefly, a 96 well coated with one specific antibody at bottom was incubated at room temperature. After washing, suitably diluted samples were added to designated wells with subsequent addition of secondary antibodies and orth-ophenylenediamine (OPD). Following an incubation period, the reaction was stopped with H_2SO_4 and the absorbance was determined at 492 nm in Multiskan ELISA plate reader (Thermo lab systems, Finland).

Real-time PCR (RT-PCR)

Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific Inc., MA, USA), and then reverse-transcribed into cDNA using the PrimeScript RT Reagent Kit (Takara Biotechnology (Dalian) CO., LTD., Dalian, China). Primers for RT-PCR were presented in *italic*. qPCR was performed with a 7500 Fast Real-Time PCR System (Applied Biosystems, MA, USA), and data were analyzed with the 2-ΔΔCT method. GAPDH expression was used as an internal control to calculate the relative expression levels of targeted genes *(Table 2)*.

The In Vitro Test

Porcine intestinal epithelial IPEC-J2 cell line was gifted by Prof. Tan (Key Laboratory of Agro-Ecological Processes in Subtropical Region, Hunan Research Center of Livestock & Poultry Sciences, South-Central Experimental Station of Animal Nutrition and Feed Science in Ministry of Agriculture, Institute of Subtropical Agriculture, the Chinese Academy of Sciences, China). Cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 [1:1] (Hyclone; Logan, Utah, USA) supplemented with 5% heat-inactivated fetal bovine serum (Invitrogen; Carlsbad, CA, USA), 1% insulin-transferrin-selenium (Invitrogen), and 1% glutamine (Sigma; St Louis, MO, USA). Cells were cultured at 37°C in a 95% air-5% CO_2 atmosphere and passaged every 72 h. IPEC-J2 cells were incubated with different doses of Met-Zn (25, 50, 75, 100 and 125 μM) or $ZnSO_4$ (25, 50, 75, 100 and 125 μM) for 24 h after the stimulation with 1 μg/mL LPS (Sigma; St Louis, MO, USA). The cells were harvested to test the expression levels of some immune factors and zinc transporters using RT-PCR.

Table 2. *Forward and reverse primers sequences of each gene*

Genes	Primer Sequence	Size
TNF-α	F:5′-TTCGGGGTGATCGGTCCCAA-3′	157
	R:5′-AGCATCTCGTGTGTTTCTGA-3′	
IL-6	F:5′-CCTGAACGACCCTACCAAG-3′	242
	R:5′- AGGCTCCATAAATGAAAGA-3′	
IL-8	F:5′-CCTGAAGACCCTACCAAG-3′	238
	R:5′-AGGCTCCATAAATGAAAGA-3′	
ZIP4	F:5′-CTGCACACACATGATGGGGA -3′	103
	R:5′-GGTTGAAAAGGCTCTCGAACA-3′	
ZIP5	F:5′-CGAGGGAACAGGACAACCA-3′	154
	R:5′-CCTATCGCCAGTCCGTCAG-3′	
ZnT1	F:5′-GAATCATTGCCACTGCTCACA-3′	115
	R:5′-GGTTGAATGGTGGTAGCGTG-3′	
GAPDH	F:5′-CTTCCTGGGCATGGAGTCCT-3′	107
	R:5′CGTGTTGGCGTAGAGGTCCTT-3′	

Statistical Analysis

Data from these experiments were analyzed by SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by a Tukey's test. Significant differences were considered with values of $P<0.05$.

RESULTS

As shown in *Table 3*, no significant difference in the lymphocyte transformation rate was observed among groups at the age of 21 days. The lymphocyte transformation rate was increased by 80 mg/kg $ZnSO_4$ and 80 mg/kg Met-Zn at the age of 35, 45 and 60 days, compared to control ($P<0.05$). But, feeding lower dosages of Met-Zn, except that 60 mg/kg Met-Zn group showed increased lymphocyte transformation rate only at the age of 35 days, had no promoting effect on the lymphocyte transformation rate. At the age of 21 and 35 days, there was no significant difference in the contents of serum globulin among groups *(Table 4)*. Feeding 80 mg/kg $ZnSO_4$ and 80 mg/kg Met-Zn to the piglets increased serum globulin content at the age of 45 and 60 days ($P<0.05$). Besides, 60 mg/kg Met-Zn increased serum globulin content at the age of 60 days ($P<0.05$).

Table 3. *Effects of Met-Zn and $ZnSO_4$ on the transformation rate of peripheral lymphocytes in weaned piglets*

Time	Control	$ZnSO_4$ 80 (mg/kg)	Met-Zn (mg/kg)				*P* Value
			20	40	60	80	
21 d	1.42±0.17	1.57±0.13	1.47±0.11	1.54±0.08	1.50±0.07	1.52±0.06	0.18
35 d	1.33±0.08^{b}	1.67±0.09^{a}	1.55±0.17^{ab}	1.53±0.13^{b}	1.56±0.14^{a}	1.57±0.12^{a}	0.02
45 d	1.35±0.06^{b}	1.45±0.07^{a}	1.31±0.03^{b}	1.35±0.11^{b}	1.37±0.08^{b}	1.47±0.04^{a}	0.03
60 d	1.37±0.12^{b}	1.65±0.13^{a}	1.36±0.06^{b}	1.37±0.23^{b}	1.43±0.17^{b}	1.68±0.10^{a}	0.04

^{a,b} values with different superscripts in the same raw are significantly different ($P<0.05$)

Table 4. *Effects of Met-Zn and $ZnSO_4$ on globulin content of weaned piglets*

Time	Control	$ZnSO_4$ (80 mg/kg)	Met-Zn (mg/kg)				*P* Value
			20	40	60	80	
21 d (g/L)	10.90±0.93	13.07±0.99	12.36±0.66	11.65±0.78	12.12±1.13	12.09±1.05	0.75
35 d (g/L)	12.30±1.13	14.87±1.37	13.15±0.88	13.56±0.93	13.62±0.84	13.57±0.73	1.01
45 d (g/L)	19.95±1.78^{b}	23.45±2.02^{a}	18.31±1.45^{b}	21.35±1.66^{ab}	22.54±1.83^{ab}	25.52±1.72^{a}	0.02
60 d (g/L)	15.67±1.24^{b}	18.65±1.19^{a}	18.36±1.72^{ab}	16.37±1.06^{b}	18.43±1.54^{a}	18.88±1.63^{a}	0.02

^{a,b} values with different superscripts in the same raw are significantly different ($P<0.05$)

Table 5. *Effects of Met-Zn and $ZnSO_4$ on the growth performance of the weaned piglets*

Items	Control	$ZnSO_4$ group	Met-Zn group	P value
Initial BW (kg)	8.52±0.04	8.52±0.09	8.53±0.06	0.95
Final BW (kg)	20.68±0.24^{b}	22.07±0.15^{a}	23.29±0.54^{a}	0.04
ADG (g)	405.33±13.73^{c}	451.51±5.53^{b}	492.13±17.26^{a}	0.03
ADFI (g)	767.26±34.27	750.26±19.10	793.60±23.34	0.12
F/G	1.89±0.03^{a}	1.66±0.03^{b}	1.62±0.02^{b}	0.21
DR (%)	4.71±0.95^{a}	1.11±0.50^{b}	2.69±1.23^{b}	0.01

^{a,b} values with different superscripts in the same raw are significantly different (P<0.05)
BW, ADFI, ADG, and F/G mean body weight, average daily feed intake, average daily gain, and feed conversion ratio, respectively

Table 6. *Effects of different dosages of Met-Zn on the expression of TNF-α, IL-6 and IL-8 in IPEC-J2 cells under the LPS stimulation*

Genes	Control	Met-Zn (μM)				
		25	50	75	100	125
TNF-α	1.00±0.12	1.75±0.28*	1.91±0.24*	2.01±0.15*	2.51±0.29**	2.56±0.21**
IL-6	1.00±0.21	1.21±0.19*	1.27±0.09*	2.03±0.18*	2.53±0.23**	2.59±0.32**
IL-8	1.00±0.09	1.97±0.31*	2.03±0.21*	2.31±0.31**	2.99±0.34**	3,27±0.18**

** P<0.05 and ** P<0.01vs. control in the same row*

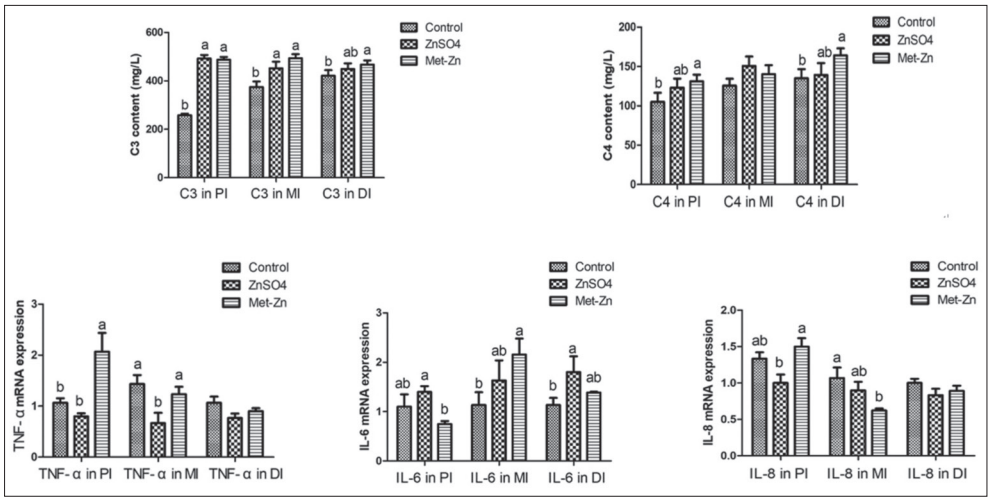

Fig 1. Effects of Met-Zn and $ZnSO_4$ on the immune-related factorsin the small intestine; ^{a,b} valueswith different superscripts in the bar are significantly different (P<0.05)

As shown in *Table 5*, the final BW and ADG were higher in the Met-Zn and $ZnSO_4$ groups than the control group (P<0.05). In addition, Met-Zn groups showed higher ADG than $ZnSO_4$ group (P<0.05). Met-Zn and $ZnSO_4$ groups showed decreased F/G (P<0.05) and DR (P<0.01) compared to control group.

Feeding Met-Zn and $ZnSO_4$ increased C3 concentrations in the anterior and middle segments of the small intestine (P<0.01, *Fig. 1)*. In addition, Met-Zn increased C3 concentrations in the posterior segment of the small intestine (P<0.01). Feeding $ZnSO_4$ showed no effect on C4 concentrations in the small intestine. However, Met-Zn increased C4 concentrations in the anterior and posterior segments of the small intestine (P<0.01). Met-Zn promoted the expression of TNF-α and IL-6 in the anterior and middle segments of the small intestine respectively (P<0.05), but inhibited the expression of IL-8 in the middle segment (P<0.05). $ZnSO_4$ promoted the expression of IL-6 in the posterior segment of the small intestine (P<0.05), but inhibited the expression of TNF-α in the middle segment (P<0.05). Under the LPS stimulation, Met-Zn and $ZnSO_4$ significantly increased the mRNA expression levels of TNF-α, IL-6 and IL-8 in IPEC-J2 in dose-dependent manners *(Table 6* and *Table 7)*

Under the LPS stimulation, Met-Zn dose-dependently increased the mRNA expression of *ZnT1* (P<0.05 or P<0.01, *Table 8)*. Met-Zn at low dosage (25 μM and 50 μM, P<0.05) and high dosage (125 μM, P<0.01) inhibited the mRNA expression of *ZIP4*. Met-Zn at dosages of 25, 50, 75, and 100 μM inhibited the expression of *ZIP5* (P<0.05), 125 μM Met-Zn promoted *ZIP5* expression (P<0.05). $ZnSO_4$ dose-dependently increased the *ZnT1*mRNA expression, but

Table 7. *Effects of different dosages of $ZnSO_4$ on the expression of TNF-α, IL-6 and IL-8 in IPEC-J2 cells under the LPS stimulation*

Genes	Control	$ZnSO_4$ (μM)				
		25	50	75	100	125
TNF-α	1.00±0.11	1.49±0.16*	1.91±0.21*	2.02±0.21*	2.76±0.24**	2.96±0.39**
IL-6	1.00±0.08	1.42±0.19*	1.25±0.17	2.24±0.27**	3.01±0.48**	3.09±0.52**
IL-8	1.00±0.15	1.46±0.20*	1.22±0.31	2.53±0.30**	2.89±0.39**	3.18±0.37**

** P<0.05 and ** P<0.01 vs. control in the same row*

Table 8. *Relative expression of target of ZnT1, ZIP4 and ZIP5 genes in the IPECs by different dosage of Met-Zn in IPECs*

Genes	Control	Met-Zn (μM)				
		25	50	75	100	125
ZnT1	1.00±0.24	1.98±0.21*	2.52±0.42*	2.48±0.23*	4.08±1.04*	7.86±2.32**
ZIP4	1.00±0.38	0.63±0.08*	0.65±0.09*	1.05±0.18	1.97±0.74	0.18±0.02**
ZIP5	1.00±0.27	0.31±0.09*	0.43±0.12*	0.24±0.11*	0.25±0.09*	2.03±0.53*

** P<0.05 and ** P<0.01 vs. control in the same row*

Table 9. *Relative expression of target of ZnT1, ZIP4 and ZIP5 genes in the IPECs by different dosage of $ZnSO_4$ in IPECs*

Genes	Control	$ZnSO_4$ (μM)				
		25	50	75	100	125
ZnT1	1.00±0.23	1.15±0.22	2.52±0.63*	3.21±1.01*	4.11±2.03*	5.42±2.83**
ZIP4	1.00±0.31	0.88±0.14	0.53±0.07*	0.33±0.09*	0.23±0.05*	0.21±0.05*
ZIP5	1.00±0.06	0.21±0.03*	0.41±0.03*	1.19±0.18	2.18±0.11*	2.67±0.14*

** P<0.05 and ** P<0.01 vs. control in the same row*

inhibited the*ZIP4* mRNA expression in a dose-dependent manner (P<0.05 or P<0.01, *Table 9)*. Low dosages of $ZnSO_4$ (25 μM and 50 μM, P<0.05) inhibited the expression of *ZIP5*, but high dosages of $ZnSO_4$ (100 μM and 125 μM, P<0.05) promoted the expression of *ZIP5*.

DISCUSSION

There is close correlation between zinc and the immune function of animals. The supplementation with zinc increased the transformation rate of peripheral lympho-cytes of piglets that lack zinc [17]. A study reported that the lack of zinc decreased the level of corticosterone in animal blood and thus influenced the immune function [18]. In this experiment, both Met-Zn and $ZnSO_4$ increased the transformation rate of peripheral lymphocytes and the content of immune globulin in piglets, which indicated that Met-Zn and $ZnSO_4$ enhance the immune function. These results are consistent with the relevant report [19], that is, adding 200 mg/kg Met-Zn in the feed increased the concentration of IgG in the piglets. Some studies also reported that zinc oxide and Met-Zn significantly increased the transformation rate of lymphocytes of piglets [20,21].

Early-weaned piglets are vulnerable to foreign pathogenic bacterium, due to a poorly developed immune function. *Escherichia coli* is such bacterium, usually causing severe diarrhea, retarded growth and even the death in early-weaned piglets, which significantly reduced the growth performance of early-weaned piglets. Complement is a group of glycoproteins that positively regulate the activity of phagocytes to eliminate pathogenic microorganism [22]. The study showed that feeding Met-Zn significantly increased the contents of C3 and C4 in the small intestine, suggesting that Met-Zn can improve the immune function of the piglets. Although feeding $ZnSO_4$ also increased C3 in the small intestine, it had no effect on the content of C4. Therefore, $ZnSO_4$ may be less effectively than Met-Zn in promoting the immune function. In line with the speculation, Met-Zn more effectively improved the growth performance, particularly the average daily gain, of the *Escherichia coli*-infected piglets than $ZnSO_4$.

Preinflammatory cytokines, including IL-6 and IL-8, play an important role in the initiation of the immune response after infection.IL-6 and IL-8 can induce the activation of macrophage, B cell and neutrophil, and the activated macrophage is able to generate TNF-α, mediating the following immune responses [23,24]. After the stimulation by the LPS of *Escherichia coli*, IL-6 and IL-8 are rapidly released, which is conducive to activating the immune system of piglets [23,24]. However, the excessive release of IL-6 and IL-8 affects the permeability of intestinal epithelium and the height of intestinal villi, leading to the down-

regulation of ion transport and digestive enzyme activity, and consequently inhibiting the digestive and absorption function. Moreover, a large amount of nutrients used for the immune response following the immune stimulation to some extent reduces the amounts of nutrients used for the growth performance. Therefore, the generation of preinflammatory cytokines should be precisely controlled to balance their effects on immune functions and growth performance. In the present study, Met-Zn and $ZnSO_4$ on the one hand stimulated the expression of some preinflammatory cytokines in some segments of small intestine; on the other hand inhibited their expression on other segments of small intestine. In addition, feeding Met-Zn and $ZnSO_4$ showed many differences in the expression of IL-6, IL-8 and TNF-α in various segments of the small intestine. These results suggested that Met-Zn and $ZnSO_4$ exerted complicated effects on the immune functions.

LPS, as the main toxic ingredient of Gram-negative bacterium, is able to induce the release of multiple inflammatory mediators from the small intestine epithelial cells [25]. In addition, the increase of zinc in the cell culture medium also promotes the expression of TNF-α, IL-6 and IL-8 [26]. In the study, under the LPS stimulation, Met-Zn and $ZnSO_4$ significantly enhanced the expression of TNF-α, IL-6 and IL-8ina dose-dependent manner. But these data were different from the results of the *in vivo* test. Zinc enters the blood circulation through intestinal mucosa, which meets the requirement of all the immune organs and thus affects the immune response throughout the body. However, in the *in vitro* test, zinc was absorbed through the zinc transporters at the cell membranes, which only affected the immune response of IPEC-J2 [19]. This likely results in the difference in the expression of TNF-α, IL-6 and IL-8 between *in vivo* and *in vitro* tests.

Immune response is accompanied with the change in the content of zinc in bodies [27-29]. Lack of zinc in cytoplasm hinders the development of precursor cells of B and T lymphocytes, reducing the immune function [30], and a high concentration of zinc in cytoplasm is highly toxic to cells [31]. Zinc transporters play important role in maintaining a reasonable level of zinc in cells and the body [32]. ZnT1 is mainly distributed in the jejunum and regulates the excretion of zinc from cells [33]. When zinc is high in surrounding environment, intestinal cells transport zinc out of cells or transport zinc to intracellular vesicles by up-regulating the mRNA expression of ZnTl, so as to reduce zinc content in the cytoplasm [34]. In this study, both Met-Zn and $ZnSO_4$ promoted the mRNA expression of ZnT1 in LPS-treated IPEC-J2 cells, which likely accelerates the efflux of zinc.

ZIP4 is located at the apical membrane of intestinal epithelial cells and mainly responsible for zinc absorption in cells. Met-Zn at low and high dosages significantly down-regulated the mRNA expression of ZIP4 in the cells under the LPS stimulation. Besides, high dosages of $ZnSO_4$ also down-regulated the ZIP4 mRNA expression. Down-regulated ZIP4 may reduce the absorption of zinc by cells and prevent toxic effect of zinc accumulation on cells. Gui et al.[35] also found that the mRNA expression of ZIP4 in IPEC-J2 cells was decreased dose-dependently by $ZnSO_4$. Han et al.[36] found that adding zinc lactate down-regulated the mRNA expression of ZIP4, and promote the proliferation of IPEC-J2. However, feeding glycine zinc to rats increased the mRNA expression level of ZIP4 in the duodenum [37].

ZIP5 is located at the basolateral side of intestinal cells and may be responsible for transferring zinc from the serosal layer to the mucous layer. Therefore, it can transport the zinc from the body to intestinal cells and finally secreted zinc from intestinal cells [38]. After ZIP5 gene is knocked out, the content of zinc in the liver of mice fed with high zinc is increased and the function of pancreas to store zinc is influenced [39]. In addition, the loss of the function of ZIP5 of intestinal cells significantly increases the content of zinc in pancreas of mice fed with high zinc and up-regulate the mRNA expression of ZIP4 in the intestinal tract [40]. In this experiment, low dosage of Met-Zn and $ZnSO_4$ down-regulated ZIP5 in IPEC-J2 cells, while high dosages of Met-Zn and $ZnSO_4$ up-regulated ZIP5.This suggests that lake of zinc in diet likely inhibits the outflow of zinc from body, while the sufficient zinc in diet likely promote the excretion of zinc from bodies.

In summary, Met-Zn showed better effects on the improvement of the growth performance and immunologic functions of early-weaned piglets than $ZnSO_4$. Met-Zn and $ZnSO_4$ showed different effect on the expression of preinflammatory cytokines in small intestine, but most similar effects on the expression of preinflammatory cytokines and zinc transporters in LPS-treated IPEC-J2 cells.

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