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Effect of dietary copper sources and concentrations on serum lysozyme concentration and protegrin-1 gene expression in weaning piglets

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Abstract

The aim of this study was to investigate the effect of dietary copper sources and concentrations on serum immune indices and the *protegrin-1* (NPG1) expression level in the bone marrow of weaning piglets. A total of 80 crossbred piglets (Duroc × Landrace × Yorkshire), with the average age of 21 days and the initial body weight of 7.00±0.03 kg, were randomly assigned to five treatments for 14 days by different kinds of diet. The dietary treatments were: 1, basal diet; 2, basal diet + 20 mg/kg Cu as $CuSO_4$; 3, basal diet + 20 mg/kg Cu as cupric citrate (CuCit); 4, basal diet + 180 mg/kg Cu as $CuSO_4$; 5, basal diet + 180 mg/kg Cu as CuCit. The results showed that compared with basal diet, supplementation with 20 mg/kg Cu as CuCit had no significant difference on growth performance (P>0.05). The incidence of diarrhoea was reduced by 71.57% and serum lysozyme concentration was increased by 170.73% (P<0.05), but there were not significant differences on serum IgA, IgG and IgM concentration (P>0.05). The mRNA expression level of NPG1 was significantly increased by 2.32-fold (P<0.01). However, the other three trial groups showed no significant differences on the experimental results compared with the basal diet group. These results indicated that supplemental 20 mg/kg Cu as CuCit could increase serum lysozyme concentration and NPG1 mRNA expression level, and reduce the incidence of diarrhoea in weaning piglets.

Introduction

It is generally demonstrated that the digestive systems are immature in the early weaning piglets. During weaning, the digestive tract of piglets must adapt to solid feed instead of sow milk. The change in the piglets' diet could result in disturbances of digestive function. An unhealthy digestive tract often causes the problem of post-weaning stress syndrome characterized by diarrhoea and seriously restricts the production potential of the piglets. Researches showed that the supplementation of 125 to 250 mg/kg copper (Cu) as $CuSO_4$ could stimulate growth rate and improve feed efficiency in weaning piglets (Bunch *et al.,* 1961; Cromwell *et al.,* 1989). It has also been observed that such high concentration of Cu supplementation can result in its high excretion in faeces (Kornegay and Harper, 1997). Recent studies have indicated that the growth stimulatory effects of organic Cu, such as cupric citrate (CuCit) (Armstrong *et al.,* 2004), cupric methionate (Huang *et al.,* 2010), cupric proteinate (Veum *et al.,* 2004) and cupric lysine (Zhou *et al.,* 1994) was an alternative to $CuSO_4$ in the piglets' diet. Moreover, CuCit has been reported to improve growth performance at lower dietary concentrations than $CuSO_4$ in broiler chickens (Ewing *et al.,* 1998). In addition, supplementation with 125 mg/kg Cu as CuCit and 250 mg/kg Cu as $CuSO_4$ were equally effective at stimulating growth and improving feed efficiency in weaning pigs (Armstrong *et al.,* 2004).

Although the mode of action of growth promoting effect on Cu remains unknown, it may be attributed to the antibacterial properties. *In vitro* studies have shown that Cu deficiency can impair the bactericidal activity of neutrophils and macrophages (Jones and Suttle, 1981; Babu and Failla, 1990). These studies provide indirect evidences for immune defense of Cu in weaning pigs.

Antimicrobial peptides (AMPs) are an important and effective component of innate immune defenses, which display direct antimicrobial activity against pathogens (Zasloff, 2002). Cathelicidins are the largest family of AMPs in pigs, which include protegrins 1 (NPG1) to 5, proline-arginine-rich 39-amino acid peptide (PR-39), prophenin 1 to 2, and porcine myeloid antimicrobial peptides 23, 36, and 37 (Kosciuczuk *et al.,* 2012). Researches have shown that cathelicidins are synthesized by bone marrow progenitor cells and cathelicidins such as, NPG1 and PR-39 are inducible (Zanetti *et al.,* 1995; Wu *et al.,* 2000). Moreover, NPG1 mRNA expression was significantly decreased after weaning in piglets, and supplemental lactoferrin in diet increased the mRNA expression level of NPG1 in pigs (Wang *et al.,* 2006; Han *et al.,* 2007). These results suggested that extrinsic modulation of porcine NPG1 expression by supplemental Cu may be possible. However, there is no available data about the regulatory effects of Cu on NPG1 expression in weaning piglets. Thus, this study aimed to investigate the effect of dietary Cu sources (CuCit *vs* $CuSO_4$) and concentrations (20 mg/kg *vs* 180 mg/kg) on serum immune indices and the NPG1 expression level in the bone marrow of weaning piglets.

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Materials and methods

Materials

In this study, CuCit and $CuSO_4$ were provided by Sichuan Animtech Feed Co., Ltd., Chengdu, China. CuCit is a kind of organic Cu, with a purity of approximately 98.5% and the content of Cu is 34.5%.

Animal care and experimental design

All procedures were approved by the Institutional Animal Care and Use Committee of Sichuan Academy of Animal Science. All the animal experiments were done according to the guidelines for animal experiments at the National Institute of Animal Health.

A total of 80 crossbred piglets (Duroc × Landrace × Yorkshire), with the average age of 21 days and the initial body weight of 7.00±0.03 kg, were randomly assigned to five groups with four replicates and four piglets (two gilts and two barrows) per pen. The piglets were raised for 14 days after 3 days adaptation. The dietary treatments were: 1, basal diet (control group); 2, basal diet + 20 mg/kg Cu as $CuSO_4$; 3, basal diet + 20 mg/kg

Cu as CuCit; 4, basal diet + 180 mg/kg Cu as $CuSO_4$; 5, basal diet + 180 mg/kg Cu as CuCit. The basal diet used in the experiment on maize-soybean meal-extruded soybean basis (Table 1), was formulated to meet the requirements for National Research Council (NRC, 2012), feeding standard of swine of China in 2004 and feed description and proximate composition of China in 2013. Piglets were housed in temperature-controlled nursery rooms and grouped in elevated pens with wire flooring. The room temperature and the relative humidity were respectively maintained 25-28°C and 50-70% throughout the experiment. Piglets were fed at 8:00, 12:00, 16:00 and 20:00 every day. During the entire experimental period, the piglets were allowed *ad libitum* access to feed and water. The nurseries were cleaned every day and disinfected every four days.

Growth and serum

Piglets were weighed individually and feed consumption per pen was measured at the beginning and end of the experiment. Feed wastage was collected each day and taken into account in the calculation of feed consumption and feed conversion efficiency. Feed conversion efficiency was calculated by the average daily gain (ADG) and the average daily feed intake (ADFI) of the piglets. At the end of the experiment, venous blood were obtained from four barrows (one pig per pen) randomly selected from each treatment for the determination of serum lysozyme and immunoglobulin concentration. Serum was obtained by centrifugation of the blood samples at 3000 rpm for 30 min at 5°C. The serum was stored at -20°C until measured by the kits purchased from Nanjing Jian Cheng Bioengineering Institute.

Incidence of diarrhoea

Severity of diarrhoea in piglets each treatment was monitored three times per day (*i.e.*, at morning, noon and evening) during the entire experimental period. A faecal consistency score was used to evaluate the severity, which was based on a scale from 0 to 3 (0 = normal faeces, 1 = soft faeces, 2 = mild diarrhoea, and 3 = watery diarrhoea). When the score was 2 or 3, diarrhoea was recorded once. Incidence of diarrhoea was calculated according to the following equation: incidence of diarrhoea = numbers of diarrhoea pigs / (numbers of pigs in treatment ×numbers of trial days) ×100%.

RNA extraction and cDNA synthesis

The barrows were slaughtered under general anaesthesia. The bone marrow from left femur of barrows was aseptically removed and immediately frozen in liquid nitrogen. The samples were stored at -80°C until they were analysed for RNA isolation. Total RNA was isolated from the bone marrow by using the RNAiso Pure RNA Isolation Kit (TAKARA BIOTECHNOLOGY CO., LTD. Dalian, China). Purified RNA samples were reverse-transcribed by using PrimeScript RT reagent Kit with gDNA Eraser for qRT-PCR (TAKARA). The process for total RNA isolation and cDNA synthesis was completed according to the manufacturer's instructions.

Quantitative real-time PCR

The NPG1 mRNA expression was assessed by quantitative real-time PCR (qRT-PCR). The qRT-PCR procedure was in accordance with the minimum information for publication of qRT-PCR experiments guidelines (Bustin *et al.,* 2009). Specific primers were designed according to the porcine NPG1 (NM_001123149.1) and *18S rRNA* (AY265350) gene sequences by using Primer Premier 5.0 (Premier Biosoft International, CA, USA) and listed in Table 2.

The qRT-PCR amplification was performed by using a total volume of 12.5 μL, containing 1 μL cDNA (50 ng/μL), 6.25 μL 2 ×SYBR Premix Ex Taq (TaKaRa), 0.5 μL 10 pM/μL of each primer and 4.25 μL ddH_2O. Reactions were amplified by using CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) and quantified by using the manufacturer's software. The thermocycling conditions comprised 3 min at 95°C and then 40 cycles with 10 s at 95°C, 30 s at 58°C, followed by a standard melting curve analysis to validate the specificity of the PCR products. All samples were amplified in triplicate from the same cDNA preparation. A 10-fold dilution series of PCR products were used to determine PCR efficiency by constructing a relative standard curve. PCR efficiencies were determined for each gene with efficiency = 100%±5%. The *NPG1* gene expression level was analysed by the 2^{-CT} method and normalized by 18S rRNA gene (Livak and Schmittgen, 2001; Wang *et al.,* 2012).

Statistical analysis

The obtained data were presented as means±standard errors (SD) and analysed by One-way ANOVA test and Tukey test. The incidence of diarrhoea of two groups was analysed using U-Test. All statistical analyses were performed by using SAS ver. 8.0 statistical package (SAS Institute, NC, USA). A P-value <0.05 was considered statistically significant.

Table 1. Composition and nutrient levels of diet on air-dry basis.

	%	Nutrient item	Nutrient levels
Ingredients			
Maize	60.17	Digestible energy, MJ·kg^{-1}	14.23
Soybean meal	12.00	Crude protein, %	20.33
Extruded soybean	15.00	Calcium, %	0.81
Fish meal	6.00	Available phosphorus, %	0.41
Wheat bran	2.50	D-lysine, %	1.19
Soybean oil	1.50	D-methionine, %	0.42
L-Lysine-Hcl	0.30	D-threonine, %	0.74
DL-Methionine	0.10	D-tryptofan, %	0.22
Threonine	0.07		
Limestone	0.95		
Calcium phosphate tribasic	0.50		
Choline chloride	0.06		
Salt	0.30		
Vitamin premix°	0.05		
Trace mineral premix #	0.50		

°Provided per kg of diet: vitamin A, 2200 U; vitamin D_3 220 U; vitamin E, 16.00 mg; vitamin K_3, 0.50 mg; vitamin B_1, 1.50 mg; vitamin B_2, 4.00 mg; vitamin B_6, 2.00 mg; niacin, 20.00 mg; calcium pantothenic, 12.00 mg; folic acid, 0.30 mg; biotin, 0.08 mg; vitamin B_{12}, 20.00 g. #Provided per kg of diet: Fe, 105.00 mg, Cu; 10.00 mg; Mn, 4.00 mg; Zn, 110.00 mg; I, 0.14 mg; Se, 0.30 mg.

Table 2. Primers used for the quantitative real-time PCR.

Gene	GenBank Accession NO.	Primer sequence (5'–3')	PCR product size, bp
protegrin-1	NM_001123149.1	Forward: CCCCAATTTTCCCGGGGCCAG Reverse: GTGAGTTGCCTGCAAATCCTTCACC	121
18S rRNA	AY265350	Forward: CTGCCTTCCTTGGATGTG	195

Results

Effects of copper on growth performance

The growth performance of weaning piglets was shown in Table 3. Compared with the control group, ADG, ADFI and the feed intake:gain (F/G) ratio did not differ when piglets receiving diets supplemented with 20 mg/kg or 180 mg/kg Cu as CuCit or $CuSO_4$ (P>0.05) throughout the study.

Effects of copper on incidence of diarrhoea

The incidence of diarrhoea of weaning piglets during the experimental period was shown in Figure 1. Compared with the control group, supplementation with different Cu sources and concentrations had reduced the incidence of diarrhoea. Moreover, supplementation with 20 mg/kg Cu as CuCit had significantly reduced the incidence of diarrhoea by 71.57% (P<0.05).

Effects of copper on serum lysozyme and immunoglobulin

The serum lysozyme and immunoglobulin concentration of weaning piglets was shown in Table 4. Compared with the control group, supplementation with 20 mg/kg Cu as CuCit and 180 mg/kg Cu as $CuSO_4$ had significantly increased serum lysozyme concentration by 170.73% (P<0.05) and 96.60% (P<0.05), respectively. No difference was observed among the trial groups of 20 mg/kg Cu as $CuSO_4$, 180 mg/kg Cu as CuCit and the control group (P>0.05). However, there was no significant difference between trial groups and the control group on serum IgA, IgG and IgM concentration (P>0.05).

Effects of copper on the *protegrin-1* gene expression

As shown in Figure 2, compared with the control group, supplementation with 20 mg/kg Cu as CuCit had significantly increased the relative mRNA expression of *NPG1* by 2.32-fold (P<0.01). However, the difference between the other three trial groups and the control group was not significant (P>0.05).

Discussion

Previous studies have demonstrated no con-

Table 3. Effects of copper on growth performance in weaning piglets.

	Control group	$CuSO_4$ 20 mg/kg	CuCit 20 mg/kg	$CuSO_4$ 180 mg/kg	CuCit 180 mg/kg
Initial weight, kg	7.038±0.035	6.985±0.016	7.006±0.018	6.976±0.027	7.012±0.015
Final weight, kg	8.480±0.187	8.553±0.225	8.784±0.209	8.656±0.182	8.650±0.195
ADG, kg	0.103±0.014	0.112±0.019	0.127±0.022	0.120±0.015	0.117±0.025
ADFI, kg	0.195±0.015	0.210±0.015	0.222±0.014	0.215±0.019	0.211±0.021
F/G, kg	1.893±0.092	1.875±0.101	1.748±0.080	1.792±0.082	1.803±0.100

CuCit, cupric citrate; ADG, average daily gain; ADFI, average daily feed intake; F/I, feed intake:gain.

Table 4. Effects of copper on serum lysozyme and immunoglobulin concentration in weaning piglets.

	Control group	$CuSO_4$, 20 mg/kg	CuCit, 20 mg/kg	$CuSO_4$, 180 mg/kg	CuCit, 180 mg/kg
Lysozyme, μg/dL	$52.07±7.09^{c}$	$54.40±3.46^{c}$	$140.97±19.90^{a}$	$102.37±14.68^{b}$	$63.60±7.48^{c}$
IgA, g/L	0.002±0.003	0.004±0.001	0.005± 0.001	0.005± 0.001	0.004±0.003
IgG, g/L	5.153±1.424	5.165±1.478	6.636±1.303	6.193±1.300	5.312±1.108
IgM, g/L	0.184±0.062	0.201±0.014	0.312±0.052	0.304±0.070	0.280±0.019

CuCit, cupric citrate. ^{a,b,c}In the same row, values with different small-letter superscripts indicate significant difference (P<0.05).

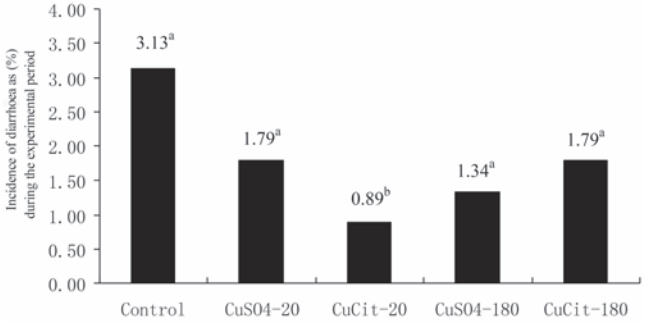

Figure 1. Effects of copper on incidence of diarrhoea in weaning piglets. Control, basal diet; CuSO4-20, basal diet + 20 mg/kg Cu as CuSO4; CuCit-20, basal diet + 20 mg/kg Cu as cupric citrate; CuSO4-180, basal diet + 180 mg/kg Cu as CuSO4; CuCit-180, basal diet + 180 mg/kg Cu as cupric citrate. Different superscripts with small letters are significantly different (P<0.05).

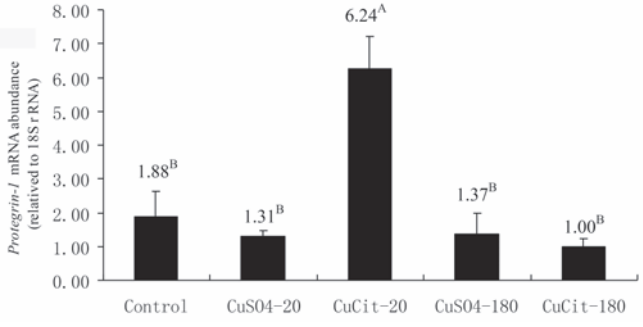

Figure 2. Effect of copper on the gene expression of protegrin-1 in weaning piglets. Control, basal diet; CuSO4-20, basal diet + 20 mg/kg Cu as CuSO4; CuCit-20, basal diet + 20 mg/kg Cu as cupric citrate; CuSO4-180, basal diet + 180 mg/kg Cu as CuSO4; CuCit-180, basal diet + 180 mg/kg Cu as cupric citrate. Each column represents the mean of 4 individual pigs±SD. Different superscripts with capital letters are significantly different (P<0.01).

sistent effect of either $CuSO_4$ or CuCit on performance in weaning piglets (Armstrong *et al.,* 2000). In the present study, compared with the control group, piglets receiving diets supplemented with 20 mg/kg or 180 mg/kg Cu as CuCit or $CuSO_4$ had no significant difference on growth performance during the first two weeks after weaning. Surprisingly, the incidence of diarrhoea was significantly reduced when piglets receiving 20 mg/kg Cu as CuCit compared with the control group. Therefore, CuCit could be used at lower concentration in diet than $CuSO_4$ without adversely affecting growth in the early weaning piglets.

Copper appears to play an important role in the body that apparently relate, among others, to the maintenance of immune function (Bonham *et al.,* 2002). Research speculated that the growth promoting effect of Cu may be due to the antibiotic-like action of Cu in the gastrointestinal tract of piglets (Bunch *et al.,* 1961). Indeed, our study showed that piglets receiving 20 mg/kg Cu as CuCit had significantly increased the concentration of serum lysozyme when compared to other piglets in the experiment. Lysozyme is one of the protein widely existed in animal blood and plays an essential role in defence against gastrointestinal pathogens and the decrease of gastrointestinal illness in weaning piglets (Nyachoti *et al.,* 2012). Moreover, lysozyme is more effective against gram positive bacteria than gram negative bacteria (Masschalck and Michiels, 2003). The participation of lysozyme in immune bacteriolysis may be in relation to serum immunoglobulin. Lysozyme could increase the rate of bactericidal activity of immunoglobulin with complement (Hill and Porter, 1974). However, in this study, there are not significant differences between trial groups and the control group on serum IgA, IgG and IgM concentration.

Protegrin-1 is a cysteine-rich, 18-residue beta-sheet peptide isolated from porcine bone marrow leukocytes, which displays antimicrobial activity against a broad range of microorganisms (Kokryakov *et al.,* 1993; Steinberg *et al.,* 1997). This study found that supplemental 20 mg/kg Cu as CuCit had significantly increased the *NPG1* mRNA abundance. Previous study has revealed that the expression of *NPG1* could be induced by lipopolysaccharide, interleukin-6, retinoic acid and *Salmonella* infection (Wu *et al.,* 2000). Our experimental results suggested that the lower concentration Cu as CuCit could regulate the expression of *NPG1* in the bone marrow of weaning piglets. Therefore, using cupric citrate to stimulate the expression of NPG1 and improve the nonspecific immune system to strengthen the host defences is a good method of protecting the weaning piglets from post-weaning stress syndrome.

Up to now, it is unclear as to why only the dietary supplementation of 20 mg/kg Cu as CuCit has the favourite results on serum lysozyme concentration and the NPG1 expression level in the bone marrow of weaning piglets. Further work is needed to study the dose response between cupric citrate and serum lysozyme concentration and NPG1 gene expression in weaning piglets.

Conclusions

In conclusion, this study has shown that supplemental 20 mg/kg Cu as CuCit could increase serum lysozyme concentration and *NPG1* mRNA expression level, and reduce the incidence of diarrhoea in weaning piglets. Our study also indicated that the lower dietary concentration Cu as CuCit was more effective than $CuSO_4$ in improving the serum immune indices and stimulating the expression of AMPs in weaning piglets. Further studies are required to confirm the immune defence of CuCit and its dietary use in order to protect the weaning piglets from post-weaning stress syndrome.

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