

# Effects of graded levels of cupric citrate on growth performance, antioxidant status, serum lipid metabolites and immunity, and tissue residues of trace elements in weaned pigs

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**Objective:** The goal of this study was to investigate the effects of cupric citrate (CuCit) on growth performance, antioxidant indices, serum lipid metabolites, serum immune indices, and tissue residues of copper (Cu), zinc, and iron in weaned pigs.

**Methods:** A total of 180 weaned pigs (Duroc×Landrace×Large White) with an average body weight of 8.98±1.21 kg were randomly assigned to a corn-soybean meal control ration, or 4 similar rations with 30, 60, 120, or 240 mg/kg Cu as CuCit. All diets contained 10 mg/kg Cu as cupric sulfate from the vitamin-mineral premix. The experiment was divided into two phases: 0 to 14 d (phase 1) and 15 to 28 d (phase 2).

**Results:** Average daily gain (ADG; linearly,  $p < 0.01$ ) and average daily feed intake (ADFI; linearly and quadratically,  $p < 0.05$ ) were affected by an increase in CuCit during phase 2. Overall period, ADG ( $p < 0.05$ ) and ADFI ( $p < 0.01$ ) were linearly increased with increasing dietary levels of CuCit. Serum malondialdehyde concentrations ( $p < 0.05$ ) and glutathione peroxidase activity ( $p < 0.01$ ) linearly decreased and increased respectively with an increase in CuCit. Serum levels of Cu-Zn superoxide dismutase were linearly affected with an increase in CuCit ( $p < 0.01$ ). Hepatic malondialdehyde levels decreased with an increase in CuCit (linearly and quadratically,  $p < 0.01$ ). Serum total cholesterol concentrations were quadratically affected ( $p < 0.05$ ) and decreased in pigs fed Cu as CuCit at 60 and 120 mg/kg and increased in pigs fed 240 mg/kg Cu as CuCit. Serum high-density lipoprotein concentrations were linearly affected with an increase in CuCit ( $p < 0.01$ ). Serum IL-1 $\beta$  levels were quadratically affected ( $p < 0.05$ ) by dietary treatment. Compared with other treatments, 240 mg/kg Cu from CuCit quadratically increased hepatic ( $p < 0.01$ ) and renal ( $p < 0.05$ ) Cu concentrations, and quadratically decreased hepatic and renal iron concentrations ( $p < 0.05$ ).

**Conclusion:** Cu administered in the form of CuCit at a dosage range of 30 to 60 mg/kg, effectively enhanced the growth performance and antioxidant status of weaned pigs.

**Keywords:** Cupric Citrate; Performance; Serum Metabolites; Antioxidant Status; Tissue Trace Elements; Weaned Pigs

## INTRODUCTION

Cupric supplementation at pharmacological doses beyond the recommendations of National Research Council has been widely applied in the feed industry to improve pig performance [1-3]. While organic copper (Cu) is more efficient than inorganic Cu in enhancing the growth performance of pigs [4,5]. Copper sulfate (CuSO<sub>4</sub>), the most common form of Cu used in pig industry, is soluble in both water and acid. Comparatively, cupric citrate (CuCit) is poorly soluble in water, but well soluble in acid and ammonia solutions, which facilitates better Cu absorption [6]. Therefore, the bioavailability of CuCit may be higher than that of CuSO<sub>4</sub>. Previous studies demonstrated that dietary CuCit supplementation enhanced growth performance in

weaned pigs at much lower doses than did CuSO<sub>4</sub> [7,8]. Thus, if lower CuCit than CuSO<sub>4</sub> dietary concentrations could be used, the growth-promoting effects of Cu could be maintained, and the environmental emission of Cu could be reduced.

Cu affects a wide spectrum of biological activities such as catalyzing the formation of hydroxyl radicals, which can lead to lipid peroxidation [9], and it plays a critical role in iron and zinc homeostasis [6,10]. In addition, pharmacological levels of Cu affected lipid metabolism in swine [11,12], and Cu intake, insufficiently or excessively, reduced immunity in experimental animals [13,14]. Comparably, the positive performance of weaned pigs was observed feeding diets containing 250 mg/kg CuSO<sub>4</sub> [3]. Previous research found that diet supplemented with 125 mg/kg Cu as CuCit stimulated growth and improved feed intake [8]. Therefore, in the present study we assumed that the optimum levels of CuCit for weaned pigs were 120 or 240 mg/kg with 10 mg/kg Cu in vitamin-mineral premix. In the meantime, we wanted to study the effects of lower dosage (30 and 60 mg/kg) of CuCit in weaned pigs. So the objective of this study was to evaluate the effects of graded levels of CuCit on growth performance, antioxidant status, serum lipid metabolites, immunity and trace elements accumulation in tissues.

## MATERIALS AND METHODS

The protocol employed in this trial was approved by the Institution of Animal Care and Use Committee of China Agricultural University. The animal experiment was conducted according to the National Institutes of Health Guidelines for the care and use of experimental animals. CuCit (98.5% CuCit and 34.4% Cu) used in this experiment was provided by Sichuan Animtech Feed Co., Ltd. (Sichuan, Chengdu, China).

### Animal, diet and experimental design

At 35 d of age, a total of 180 weaned pigs (Duroc×Landrace×Large White) with an average body weight of 8.98±1.21 kg were assigned by sex and body weight to five treatments with six replicates per treatment and six pigs (three barrows and three gilts) per pen in a randomized complete block design. They were treated with gradient doses of Cu as CuCit (0, 30, 60, 120, and 240 mg/kg; Table 1). Additionally, all diets contained 10 mg/kg Cu as CuSO<sub>4</sub> from a vitamin-mineral premix and corn meal was replaced with CuCit in the other four treatments. Pigs were placed in concrete floor pens (1.2×2.0 m) partially slatted with steel, and each pen was equipped with a self-feeder and a nipple drinker. Pigs had *ad libitum* access to both feed and water. Diets were in a mash form and formulated to meet or exceed NRC [1] requirements in two phases: 0 to 14 d (phase 1) and 15 to 28 d (phase 2). Cu concentrations in experimental diets, analyzed and presented in Table 2, were as described by Armstrong et al [8].

**Table 1.** Composition and chemical composition of the basal diets (as-fed basis)<sup>1,2)</sup>

Items	Phase 1	Phase 2
Ingredient (%)		
Corn meal	55.00	58.76
Soybean meal	14.32	18.27
Soybean oil	1.97	2.00
Extruded soybeans	10.65	7.05
Fish meal	3.00	3.00
Soy protein concentrate	5.10	1.35
Whey powder	6.00	6.00
Dicalcium phosphate	1.46	1.24
Limestone	0.53	0.42
Salt	0.25	0.25
L-lysine-HCl	0.53	0.52
L-threonine	0.18	0.17
L-tryptophan	0.02	0.02
DL-methionine	0.29	0.25
Choline chloride (50%)	0.20	0.20
Vitamin-mineral premix <sup>3)</sup>	0.50	0.50
Total	100.00	100.00
Nutrient levels <sup>4)</sup> (%)		
Digestible energy (kcal/kg)	3,542	3,490
Crude protein	20.56	18.87
Lysine	1.53	1.40
Methionine	0.58	0.52
Threonine	0.95	0.88
Calcium	0.80	0.70
Total phosphorus	0.65	0.60

<sup>1)</sup> Corn meal was replaced with CuCit in the other four treatments.

<sup>2)</sup> Phase 1 basal diet analyzed 15.47 mg copper, 148.68 mg zinc, and 163.83 mg iron per kilogram of diet; Phase 2 basal diet analyzed 15.87 mg copper, 140.95 mg zinc, and 165.16 mg iron per kilogram of diet.

<sup>3)</sup> Provided (per kilogram of diet): vitamin A, 12,000 IU; vitamin D<sub>3</sub>, 2,000 IU; vitamin E, 30 IU; vitamin K<sub>3</sub>, 2.5 mg; vitamin B<sub>1</sub>, 2.5 mg; vitamin B<sub>2</sub>, 4 mg; vitamin B<sub>6</sub>, 3 mg; vitamin B<sub>12</sub>, 20 µg; niacin, 40 mg; pantothenic acid, 12.5 mg; folic acid, 0.7 mg; biotin, 0.07 mg; iron, 100 mg (as iron sulfate monohydrate); copper, 10 mg (as copper sulfate); zinc, 80 mg (as zinc sulfate); manganous, 30 mg (as manganous oxide); iodate, 0.25 mg (as calcium iodate); selenite, 0.15 mg (as sodium selenite).

<sup>4)</sup> All data are analyzed values except for digestible energy.

### Performance measurements and blood sampling

On d 0, 14, and 28 of the experiment, body weight and daily feed consumption were measured for the calculation of average daily gain (ADG), average daily feed intake (ADFI), and gain-to-feed ratio (G:F). On d 28, six pigs (three barrows and three gilts) from each treatment (one pig per pen) were selected based on the mean body weight of each pen. Blood samples were collected from the precaval vein after 12 h fasting and allowed to clot at room temperature of 25°C for 20 min and centrifuged at 3,000×g for 10 min at 4°C. The serum was then removed and

**Table 2.** Analyzed copper concentrations in experimental diets (as-fed basis)

Item	Cupric citrate additional content (mg/kg)				
	0	30	60	120	240
Analyzed values of copper (mg/kg)					
Phase 1, 0 to 14 d	15.47	46.63	78.11	134.97	256.71
Phase 2, 15 to 28 d	15.87	48.62	75.31	136.88	255.02

stored at  $-20^{\circ}\text{C}$  until assayed.

### Liver and kidney sampling

On d 28 of the experiment, 36 pigs with body weights approaching the mean value of pigs in each pen were selected. The liver of the lobe adjacent to the gallbladder and kidney were rinsed in 0.01 mol/L phosphate-buffered saline (phosphate buffer saline, pH 7.4) and snap frozen in liquid nitrogen. The liver samples were stored at  $-20^{\circ}\text{C}$  to determine the levels of Cu, iron (Fe), zinc (Zn), malondialdehyde (MDA), and the activity of Cu-Zn superoxide dismutase (Cu/Zn-SOD), glutathione peroxidase (GSH-Px), and ceruloplasmin (CP). The kidney samples were stored at  $-20^{\circ}\text{C}$  for trace element analysis (Cu, Fe, and Zn).

### Antioxidant indices

Liver samples were homogenized in 0.1 mol/L tris (hydroxymethyl) aminomethane buffer at  $4^{\circ}\text{C}$ , pH 7.4, to make a 10% (w/v) homogenate, using a polytron homogenizer for 5 min and a sonic homogenizer for 3 min. The homogenates were centrifuged at  $3,000\times g$  for 5 min at  $4^{\circ}\text{C}$ , and the supernatants were collected and stored at  $-20^{\circ}\text{C}$  for enzyme analysis.

The MDA levels in the serum and liver were determined with 2-thiobarbituric acid at 532 nm. Serum and liver Cu/Zn SOD activity was determined at 550 nm. GSH-Px activity was determined at 412 nm. CP activity was measured at 450 nm. All assays using commercial kits (Jiancheng Biochemical Reagent Co., Nanjing, China) were performed according to the manufacturer's instructions.

### Serum lipid metabolites

The concentrations of serum total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were determined at 546 nm. Triglyceride (TG) levels were detected at 505 nm. Very-low-density lipoprotein cholesterol (VLDL-C) levels were detected at 450 nm. All assays followed the instructions of commercial kits (BioSino Bio-technology and Science Inc., Beijing, China) using an automatic biochemical analyzer (Hitachi 7160, Hitachi High-Technologies Corporation, Tokyo, Japan).

### Serum immune indices

The concentrations of serum immunoglobulin A (IgA), immunoglobulin M (IgM) were determined at 340 nm, immunoglobulin G (IgG) levels were determined at 700 nm, interleukin-1 beta (IL-1 $\beta$ ) and interferon gamma (INF- $\gamma$ ) levels were measured at 450 nm. All assays followed the instructions of commercial kits (Beijing Sino-UK Institute of Biological Technology, Beijing, China) using an automatic biochemical analyzer (Hitachi 7160, Hitachi High-Technologies Corporation, Tokyo, Japan).

### Tissue trace elements

Liver and kidney samples were uniformly cut from tissues, wet-

digested using nitric-perchloric acid and then diluted with deionized-distilled water for mineral analyses [15]. The levels of serum, hepatic and renal Cu, Fe, and Zn were measured by using inductively coupled plasma mass spectrometry (7500, Agilent Technologies, Inc., Santa Clara, CA, USA) as described by Huang et al [16].

### Statistical analysis

Data were analyzed by one-way analysis of variance according to the general linear model procedures of SAS 9.2 (SAS Institute Inc., Cary, NC, USA) as a randomized complete block design by weight, which included terms for treatments and blocks. Each pen served as the experimental unit for growth performance, and individual pig was considered as the experimental unit for the other indices. Coefficients for unequally spaced contrasts were generated by interactive matrix algebra procedure (IML) of SAS, after which the linear and quadratic responses of the Cu level as CuCit were assessed by the orthogonal polynomial contrast. Significance was declared at  $p<0.05$ .

## RESULTS

### Growth performance

The growth performance in weaned pigs from each treatment is presented in Table 3. During phase 2, ADG (linearly,  $p<0.01$ ) and ADFI (linearly and quadratically,  $p<0.05$ ) were affected with increasing dietary levels of CuCit in weaned pigs. Overall period, ADG ( $p<0.05$ ) and ADFI ( $p<0.01$ ) increased linearly with increasing CuCit, but ADG and G:F in phase 1, G:F in phase 2, and the overall period were not affected by Cu levels.

### Antioxidant status

Serum MDA concentrations ( $p<0.05$ ) and GSH-Px activity ( $p<0.01$ ) linearly decreased and increased, respectively, with an increase in CuCit (Table 4). Serum Cu/Zn-SOD activity was linearly affected ( $p<0.01$ ) by an increase in CuCit. Hepatic MDA levels linearly ( $p<0.01$ ) and quadratically ( $p<0.01$ ) decreased with an increase in CuCit. Serum CP activity and hepatic Cu/Zn-SOD, GSH-Px, and CP activity was unaffected by Cu addition.

### Serum lipid metabolites

As shown in Table 5, serum TC concentrations were quadratically affected ( $p<0.05$ ) by CuCit with decreasing observed in pigs fed Cu as CuCit at 60 and 120 mg/kg, which then increased for pigs fed the 240 mg/kg treatments. Serum HDL-C concentrations were linearly affected ( $p<0.01$ ) with an increase in CuCit. Serum HDL-C concentrations were linearly affected with an increase in CuCit ( $p<0.01$ ). Serum TG, LDL-C, and VLDL levels were not affected by Cu levels.

### Serum immune indices

The effects of CuCit on serum immune indices in weaned pigs

**Table 3.** Effects of cupric citrate on growth performance in weaned pigs

Item	Copper as cupric citrate (mg/kg)					SEM	p-value		
	0	30	60	120	240		ANOVA	Linear	Quadratic
Phase 1, 0 to 14 d									
Average daily gain (g)	307	359	380	383	400	22	0.070	0.250	0.888
Average daily feed intake (g)	515	609	676	668	702	36	0.010	0.089	0.880
Gain-to-feed ratio	0.595	0.590	0.563	0.574	0.568	0.010	0.118	0.223	0.953
Phase 2, 15 to 28 d									
Average daily gain (g)	452	509	511	468	438	15	0.005	0.002	0.063
Average daily feed intake (g)	832	936	928	863	781	30	0.008	0.007	0.031
Gain-to-feed ratio	0.544	0.545	0.550	0.545	0.561	0.011	0.772	0.988	0.380
Overall period, 0 to 28 d									
Average daily gain (g)	380	434	446	426	419	16	0.084	0.023	0.323
Average daily feed intake (g)	673	773	802	767	742	26	0.029	0.009	0.161
Gain-to-feed ratio	0.563	0.563	0.555	0.556	0.564	0.009	0.874	0.845	0.346

SEM, standard error of the mean; ANOVA, analysis of variance.

Data are the means of six replicates of six pigs (three barrows and three gilts) per pen.

are shown in Table 6. Serum IL-1 $\beta$  levels were quadratically affected ( $p < 0.05$ ) by dietary treatment. There were no significant differences in serum IgM, IgA, IgG, or IFN- $\gamma$  levels among all treatments.

#### Tissue trace elements

Table 7 shows the effects of CuCit on the concentrations of tissue trace elements in weaned pigs. There were no significant differences in serum Cu, Fe, or Zn levels among all treatments.

Compared with other treatments, 240 mg/kg Cu as CuCit quadratically increased hepatic ( $p < 0.01$ ) and renal ( $p < 0.05$ ) Cu concentrations, and quadratically decreased hepatic and renal Fe concentrations ( $p < 0.05$ ). Hepatic and renal Zn levels were not affected by Cu levels.

## DISCUSSION

According to the results of present study, ADG and ADFI were

**Table 4.** Effects of cupric citrate on antioxidant status in weaned pigs

Items	Copper as cupric citrate (mg/kg)					SEM	p-value		
	0	30	60	120	240		ANOVA	Linear	Quadratic
Serum									
Malondialdehyde (nmol/mL)	9.50	7.35	7.24	7.94	8.14	0.47	0.021	0.007	0.232
Copper-zinc superoxide dismutase (U/mL)	48.48	50.49	50.89	48.78	48.20	0.41	<0.001	<0.001	0.112
Glutathione peroxidase (U/mL)	186.54	207.72	215.74	205.41	199.12	5.55	0.017	0.003	0.133
Ceruloplasmin (U/mL)	45.78	48.43	48.53	47.91	51.07	2.18	0.570	0.629	0.524
Liver									
Malondialdehyde (nmol/mL)	6.10	3.40	3.46	4.51	6.10	0.40	<0.001	<0.001	0.001
Copper-zinc superoxide dismutase (U/mL)	30.57	28.13	27.91	29.28	29.13	0.92	0.292	0.058	0.682
Glutathione peroxidase (U/mL)	405.74	333.26	337.01	346.58	375.52	28.43	0.357	0.154	0.242
Ceruloplasmin (U/mL)	21.75	19.78	19.04	19.43	22.38	1.59	0.312	0.184	0.090

SEM, standard error of the mean; ANOVA, analysis of variance.

Data are the means of six replicates of one pig per pen.

**Table 5.** Effects of cupric citrate on serum concentrations of lipid metabolites in weaned pigs

Items	Copper as cupric citrate (mg/kg)					SEM	p-value		
	0	30	60	120	240		ANOVA	Linear	Quadratic
Total cholesterol (mmol/L)	1.80	1.81	1.53	1.60	1.97	0.10	0.035	0.093	0.022
Triglyceride (mmol/L)	0.45	0.52	0.46	0.47	0.33	0.05	0.141	0.550	0.057
High-density lipoprotein (mmol/L)	0.50	0.50	0.67	0.51	0.46	0.04	0.014	0.003	0.439
Low-density lipoprotein (mmol/L)	0.94	1.04	0.83	0.83	1.02	0.06	0.051	0.427	0.072
Very-low-density lipoprotein (mmol/L)	52.01	55.80	55.30	43.84	54.60	5.21	0.480	0.311	0.196

SEM, standard error of the mean; ANOVA, analysis of variance.

Data are the means of six replicates of one pig per pen.

**Table 6.** Effects of cupric citrate on serum immunity in weaned pigs

Items	Copper as cupric citrate (mg/kg)					SEM	p-value		
	0	30	60	120	240		ANOVA	Linear	Quadratic
Immunoglobulin M (g/L)	1.18	1.07	0.92	1.02	0.96	0.12	0.568	0.248	0.943
Immunoglobulin A (g/L)	1.82	1.96	1.96	1.66	1.74	0.14	0.482	0.178	0.754
Immunoglobulin G (g/L)	9.86	9.43	9.05	8.63	8.06	0.66	0.383	0.967	0.772
Interleukin-1 beta (pg/mL)	56.81	60.09	46.98	59.37	47.96	3.72	0.050	0.090	0.043
Interferon gamma (pg/mL)	105.88	115.18	127.83	116.64	114.58	8.53	0.513	0.116	0.686

SEM, standard error of the mean; ANOVA, analysis of variance. Data are the means of six replicates of one pig per pen.

linearly affected with increasing dietary levels of CuCit, and lower dosages (30 and 60 mg/kg) of CuCit showed a better growth performance over the experimental period from d 15 to 28 and overall period. Comparably, the dose range of CuSO<sub>4</sub>, which significantly improved growth performance in pigs was typically higher at 100 to 250 mg/kg [3,17,18]. Furthermore, another study predicted that peak ADG and ADFI in weaned pigs were obtained with supplemental Cu levels at 150 and 114 mg/kg, respectively, in the form of CuSO<sub>4</sub> [19]. However, it should be noted that our results differ from those observed in Armstrong et al [8,20], in which piglets (pig improvement company) or crossbred piglets weaned at 18 d were fed CuCit. Armstrong et al [8] reported that 33 to 100 mg/kg Cu from CuCit did not affect the performance of piglets, but 125 mg/kg Cu as CuCit stimulated growth and improved feed intake. The differing effects of Cu may due to the difference among weaning age and body weight of pigs. Pigs weaned at 18 d of age may have lower absorption efficiency of Cu since they have less Cu transport receptors in their small intestine. Therefore, the requirements of CuCit which improved the performance of weaned pigs may be lower in our research. In addition, the results in present study showed that the growth performance of piglets during d 0 to 14 was little affected by diets supplementation with CuCit which may be due to the weaning stress.

There are complex systems in which Cu trafficking evolves to satisfy the cellular requirements while minimizing toxicity [21]. Apart from promoting growth performance in pigs, lower CuCit dosage directly resulted in fewer residues in tissues. In the present study, only the 240-mg/kg CuCit dose caused a significant Cu increase in the serum, liver and kidneys. The quadratic rather than linear increase in hepatic and renal Cu concentrations may be related to the large gap between the 120 and 240 mg/kg CuCit treatments. Several previous reports also suggested that tissue Cu levels increased when pigs were fed high levels of Cu above 120 mg/kg, regardless of organic or inorganic sources [2,20]. Studies showed that pigs fed Cu at increased levels to 150 mg/kg had a significant increase in plasma Cu levels [22]. Liver Cu levels were significantly increased by copper chelate of 2-hydroxy-4-methylthiobutanoic acid (Cu at 170 mg/kg) compared with the control [5]. Similarly, pigs fed tribasic copper chloride (TBCC; 225 mg/kg) had higher liver Cu concentrations, compared with the control and those fed Cu at 225 mg/kg as CuSO<sub>4</sub> [16]. The failure of the doses of CuCit below 120 mg/kg to increase the Cu levels in the serum, liver, and kidneys may be a result of the liver regulation function: most of the newly absorbed Cu was taken up by the liver, and excessive Cu in the liver was excreted to bile [23].

High levels of Cu caused oxidative damage and lipid perox-

**Table 7.** Effects of cupric citrate on the concentrations of tissue trace element in weaned pigs

Items	Copper as cupric citrate (mg/kg)					SEM	p-value		
	0	30	60	120	240		ANOVA	Linear	Quadratic
Serum (mg/L)									
Copper	1.71	1.84	2.00	2.02	2.38	0.11	0.007	0.675	0.123
Iron	10.03	10.18	8.40	8.47	7.74	0.74	0.118	0.586	0.635
Zinc	3.25	3.33	2.53	2.98	1.88	0.72	0.605	0.727	0.349
Liver (mg/kg)									
Copper	6.22	6.49	6.67	8.11	23.11	1.54	<0.001	0.074	<0.001
Iron	85.96	90.35	90.60	82.18	39.53	8.84	0.003	0.156	0.006
Zinc	92.33	84.13	90.12	71.91	64.61	8.33	0.124	0.353	0.859
Kidney (mg/kg)									
Copper	8.67	7.41	8.25	7.66	12.11	1.08	0.038	0.489	0.010
Iron	38.21	38.74	44.75	45.04	34.64	3.30	0.157	0.284	0.041
Zinc	22.57	21.68	21.13	21.89	21.98	0.62	0.597	0.134	0.698

SEM, standard error of the mean; ANOVA, analysis of variance. Data are the means of six replicates of one pig per pen.

idation, because Cu (II) ions catalyzed the formation of hydroxyl radicals, which can lead to lipid peroxidation [9]. As the component of several enzymes such as Cu/Zn-SOD [24], Cu at an appropriate dosage may decrease lipid peroxidation by increasing the activity of antioxidant enzymes [25,26]. The degree of lipid peroxidation could be evaluated by MDA [27]. Reports about the effects of pharmacological Cu in feed on tissue MDA concentrations focused on the duodenum rather than the serum and liver [16,28]. A previous study showed that duodenal MDA contents were higher when pigs were fed 225 mg/kg supplemental Cu as CuSO<sub>4</sub> rather than TBCC [16]. In our trial, increasing doses of CuCit led to linear and quadratic increases in serum and hepatic MDA concentrations. And Cu supplemented at doses between 30 and 120 mg/kg as CuCit led to lower serum and hepatic MDA concentrations, which was in accordance with the greater serum Cu/Zn-SOD and GSH-Px activity. Feng et al [29] found 100 mg/kg Cu increased erythrocyte Cu/Zn-SOD activity compared with that in control pigs [29]. Fry et al [28] found that pigs fed 225 mg/kg CuSO<sub>4</sub> had lower serum GSH-Px activity compared with that in the control treatment [28]. The lower MDA levels, higher Cu/Zn-SOD and GSH-Px activity observed in our study may be attributed to the lower Cu concentrations in tissues when pigs were fed 30 to 60 mg/kg Cu as CuCit. Thus, low dosages of Cu may decrease the formation rate of oxygen free radicals, which may increase the antioxidant levels of weaned pigs, while high Cu levels may have the opposite effect. Our research indicated the effects of dietary CuCit on hepatic oxidative stress tended to fit the hormesis curve, where hepatic MDA levels initially decreased and then increased with increasing CuCit supplementation.

There were limited reports about the effects of dietary Cu addition on serum lipid metabolites of pigs. A study suggested 100 mg/kg Cu as CuCit improved serum TC levels in weaned pigs [30], however, serum TC concentrations were highest in pigs fed the 240 mg/kg treatments. Serum HDL-C concentrations linearly increased in pigs fed CuCit at 60 mg/kg, but these treatments had no effect on TG, LDL-C, and VLDL-C levels in our trial. Li et al [31] found no Cu treatment differences were observed in plasma TC, TG, HDL-C, and LDL-C levels [31]. Therefore, the effects of Cu as CuCit on lipid metabolites in weaned pigs are inconclusive.

Cu plays an important role in maintaining the immune function of pigs, and its deficiency or excess intake could impair immune cell numbers and functions [32]. Some studies showed that interleukin-2 (IL-2) was reduced by Cu deficiency, which possibly decreased T cell proliferation [14,33]. Appropriate serum concentrations of immunoglobulins, IL-1 $\beta$ , and INF- $\gamma$ , which are fundamental components of the immune system, indicate the activation of an immune response [34]. In our research, serum levels of IgM, IgA, IgG, and INF- $\gamma$  showed no differences among all treatments. The results were similar to recent research where 20 and 180 mg/kg Cu as CuCit had no effects on serum

IgA, IgM, and IgG levels in piglets [35]. Although serum IL-1 $\beta$  levels were quadratically affected by dietary treatments in our trial, it did not indicate that CuCit had improved the serum immunity in weaned pigs. Considering the definitive mechanisms of the effects of CuCit on immunity in weaned pigs, further research is warranted.

Divalent metal transporter 1 and copper transport receptor 1 are involved in the active transport of Cu (II), Fe, and Zn (II) [36]. Thus, the absorption and utilization of Cu might be related to Fe and Zn. In addition, under certain circumstances, a mucosal block to metal absorption may occur via sequestration of Fe and Cu by ferritin and metallothionein, respectively [37,38]. In our study, pigs fed Cu at 240 mg/kg from CuCit quadratically decreased hepatic and renal Fe concentrations. There were no significant differences in serum Fe or Zn, hepatic and renal Zn levels among all treatments. Huang et al [16] indicated that hepatic Fe and Zn levels were lower and higher, respectively, in pigs fed 225 mg/kg Cu as TBCC than those in the control and those fed 225 mg/kg Cu as CuSO<sub>4</sub> [16]. The results were similar to those of Fry et al [28] which showed that hepatic Fe concentrations were less in TBCC pigs compared with the control group [28]. Whereas Cu lysinate (250 mg/kg) decreased Zn solubility in the digestion of broilers, supplemental CuSO<sub>4</sub> and TBCC did not [39]. This may indicate that diets supplemented below 120 mg/kg Cu from CuCit had no effects on serum and hepatic Fe or Zn levels when dietary Fe and Zn concentrations were supplemented above the dietary requirements in this experiment.

## CONCLUSION

Cupric citrate (30 to 60 mg/kg) is an efficient copper supplement due to a relative low supplemental dosage required to maintain a high level of growth performance, antioxidant status and low tissue residues.

## CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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