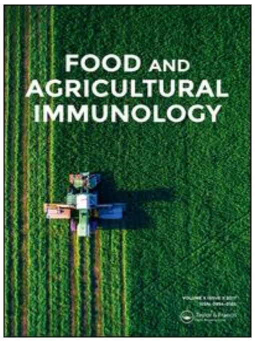

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Effect of dietary copper source (inorganic *vs.* chelated) on immune response, mineral status, and fecal mineral excretion in nursery piglets

Peng Liao [a], Xugang Shu[b], Min Tang[c], Bie Tan[a] and Yulong Yin[a]

[a]Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha, People's Republic of China; [b]College of Chemistry and Chemical Engineering, Zhongkai University of Agriculture and Engineering, Guangzhou, People's Republic of China; [c]Department of Gynaecology and Obstetrics, Mawangdui District of Hunan Provincial People's Hospital, Changsha, People's Republic of China

ABSTRACT

This experiment was conducted to determine the effect of dietary supplementation with copper sulfate and cupreous N-carbamylglutamate chelate (NCG-Cu) on the growth performance, serum biochemical profile, immune response, tissue mineral distributions, and fecal excretion of minerals in nursery piglets. Eighteen healthy nursery piglets were randomly assigned to 3 dietary treatments consisting of no copper in either form (control), 650 g/t copper sulfate (650 g/t Cu) or 320 g/t NCG-Cu (320 g/t NCG-Cu) for 35 days. Pigs fed the 320 g/t NCG-Cu diet showed a significantly ($P < 0.05$) elevated growth rate, feed conversion efficiency, IgA and IgM levels, and decreased diarrhea rate compared to those fed the 650 g/t Cu diet. Fecal copper (Cu) and zinc (Zn) were increased ($P < 0.05$) when pigs were fed the 650 g/t Cu diets compared with those fed the 320 g/t NCG-Cu diets. Tissue Cu has limited effects on tissue mineral distribution, except for the distribution in the spleen and liver ($P < 0.05$). These results indicated that 320 g/t NCG-Cu (chelated) was as effective as 650 g/t Cu (inorganic Cu) for stimulating growth and the immune response and reducing dietary fecal Cu excretion, thus reducing environmental pollution.

Abbreviations: NCG: N-carbamylglutamate chelate; ADG: average daily gain; ADFI: average daily feed intake; F/G: feed to gain ratio; ALB: albumin; GLU: blood glucose; CREA: creatinine; ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate amino transferase; GLB: globulin; TC: total cholesterol; TP: total protein; urea: Urea; D-BIL: direct-acting-bilirubin; T-BIL: total bilirubin; UA: urate; CK: creatine kinase; LDH: lactate dehydrogenase; IgG: immunoglobulin G; IgA: immunoglobulin A; IgM: immunoglobulin M; C_3: complement C_3; LD: longissimus dorsi; ICP-OES: inductively

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CONTACT Peng Liao liaopeng@isa.ac.cn Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha 410125, People's Republic of China; Min Tang enid0568@163.com Department of Gynaecology and Obstetrics, Mawangdui District of Hunan Provincial People's Hospital, Changsha 410016, People's Republic of China; Yulong Yin yinyulong@isa.ac.cn Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha 410125, People's Republic of China

coupled plasma optical emission spectrometry; AA: amino acid; GIT: gastrointestinal; Cu-Met: Cu-methionine; CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber.

1. Introduction

Micromineral copper (Cu) is an essential mineral that drives a wide array of biochemical programmes that are important for life, including normal organ growth and development and immune system function in young mammals (Bauerly, Kelleher, & Lonnerdal, 2005; Brubaker & Sturgeon, 1956; Fry et al., 2012; Liao et al., 2017). Modern commercial industrial pig production involves dietary supplementation with different amounts of Cu from different sources, which is associated with significant growth promotion as well as environmental and immunological impacts on pigs at different stages of development (Fry et al., 2012; Huang et al., 2015; Liao et al., 2017). Studies has demonstrated that Cu supplementation at rates from 100 to 250 mg/kg diet can promote growth and feed intake and reduce the fecal excretion of Cu in swine (Armstrong, Cook, Ward, Williams, & Spears, 2004; Cromwell, Lindemann, Monegue, Hall, & Orr, 1998; Fry et al., 2012; Liao et al., 2017).

Two major sources of Cu are used in the swine industry, organic and inorganic Cu minerals, which show different levels of bioavailability in animals (Acda & Chae, 2002; Brubaker & Sturgeon, 1956; Gonzales-Eguia, Fu, Lu, & Lien, 2009; Liao et al., 2017; Pluske, Pethick, Hopwood, & Hampson, 2002). Some studies have found that organic Cu minerals show increased bioavailability and absorption, which results in improved growth performance, compared with inorganic Cu mineral sources (Apgar, Kornegay, Lindemann, & Notter, 1995; Beames & Lloyd, 1965; Coffey, Cromwell, & Monegue, 1994; Hill et al., 1983; Liao et al., 2017; Van Heugten & Coffey, 1992; Zhou, Kornegay, Lindemann, et al., 1994), but other studies have not (Acda & Chae, 2002; Apgar & Kornegay, 1996; Kegley & Spears, 1994; Pluske et al., 2002). Another strategy for reducing Cu mineral concentrations in diets is the inclusion of sources that may exhibit greater bioavailability than commonly used chelated forms (Creech et al., 2004; Liao et al., 2017). Previous studies have evaluated the acute and sub-acute toxicity and the mutagenicity of N-carbamylglutamate (NCG) and cupreous NCG chelate (NCG-Cu) (Wan et al., 2015; Wu, Wan, Xie, Li, et al., 2015), and subsequent study has indicated that dietary supplementation with a chelate form of NCG-Cu at 640 g/t can stimulate growth and immune function and reduce fecal Cu excretion in weanling pigs more than with 650 g/t $CuSO_4$ (Liao et al., 2017). Under normal physiological conditions and with adequate intake, pigs need different amounts of Cu at different development periods (Fry et al., 2012; Huang et al., 2015; Tian et al., 2001). Whether NCG-Cu can be used as a dietary supplement at 50% of the concentration of $CuSO_4$ used in the nursery period to maintain the growth-promoting and immune function effects of Cu while reducing its fecal excretion has not been evaluated in pigs during the nursery period.

Therefore, this study was designed to evaluate the effectiveness of dietary supplementation with $CuSO_4$ and NCG-Cu on the growth performance, serum biochemical profiles, immune response, tissue mineral levels and fecal excretion of minerals in nursery piglets.

2. Materials and methods

2.1. Ethics statement

The experimental design and procedures used in this study were approved by the Animal Care and Use Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences (Changsha, Hunan Province, China, ISACAS Protocol # 2014ISA0607).

2.2. Pig management

Eighteen Yorkshire × (Duroc × Landrace) nursery pigs weighing 10.30 ± 0.13 kg each were stratified by body weight (BW) and randomly allotted into 3 dietary treatments: 1) control (basal diet without added Cu, analyzed as 10.24 mg Cu/kg diet), 2) 650 g/t Cu (basal diet + 650 g/t $CuSO_4$, analyzed as 160 mg Cu/kg diet), and 3) 320 g/t NCG-Cu (basal diet + 320 g/t NCG-Cu, analyzed as 80 mg Cu/kg diet) (Tanke Bio-Technology Co., Ltd., Guangdong Province, China). Each dietary treatment contained six replicates ($n = 6$), and pigs were raised individually in cages. Diets were isoenergetic and met the nutritional requirements for pigs according to the National Research Council (NRC, 2012) (Table 1) (NRC, 2012; Wu, Liao, He, Feng, et al., 2015), and pigs were offered the diets and water *ad libitum*. The experiment lasted for 35 d, and results related to BW and feed consumption, such as the average daily gain (ADG), average daily feed intake (ADFI), the feed to gain ratio (F/G), and diarrhea rate, were recorded at the beginning and end of the experimental period as previously described (Duan et al., 2014; Liao et al., 2017; Wu, Liao, He, Feng, et al., 2015; Wu, Liao, He, Ren, et al., 2015). The diarrhea rate was calculated as follows

Table 1. Compositions and nutrient levels in basal diets (as-fed basis).

Ingredients	Contents (%)	Calculated and analyzed nutrient composition[b]	Contents
Corn (43%CP)	63.70	DE (MJ/kg)[c]	14.60
Soybean meal	19.80	CP	20.27
Whey powder	4.30	Total Ca	0.69
Fish meal (64%CP)	9.00	Total P	0.57
Soybean oil	0.80	Starch	40.22
Lysine hydrochloride	0.38	NDF	8.54
Hydroxy methionine	0.10	ADF	3.29
L-threonine	0.09	Lys	1.26
L-tryptophan	0.01	Met + Cys	0.62
$CaHPO_3$	0.00	Thr	0.76
Rock-powder	0.52	Trp	0.20
Salt	0.30	Arg	1.09
1% Premix[a]	1.00	His	0.44
Total	100.00	Ile	0.71
EAA	7.91	Leu	1.52
NEAA	9.74	Phe	0.81
EAA/NEAA	0.80	Val	0.72

Notes: CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber.
[a]Premix provided the following amounts of vitamins and minerals per kilogram on an as-fed basis: vitamin A, 10,800 IU; vitamin D_3, 4,000 IU; vitamin E, 40 IU; vitamin K_3,4 mg; vitamin B_1, 6 mg; vitamin B_2, 12 mg; vitamin B_6, 6 mg; vitamin B_{12}, 0.05 mg; biotin, 0.2 mg; folic acid, 2 mg; niacin, 50 mg; D-calcium pantothenate, 25 mg; Fe, 100 mg as ferrous sulfate; Mn, 40 mg as manganese oxide; Zn, 100 mg as zinc oxide; I, 0.5 mg as potassium iodide; and Se, 0.3 mg as sodium selenite. The values are expressed as a percentage (%) except for digestible energy (DE; MJ/kg) and essential amino acids (EAAs) / nonessential amino acids (NEAAs).
[b]All other values represent analyzed values.
[c]The DE was calculated according to NRC (2012).

(Liao et al., 2017): diarrhea rate (%) = {piglets with diarrhea [n] / (total piglets in the experiment [n] × duration of the experiment [d])} × 100%.

2.3. Sample collection

At the end of each experiment, the 6 pigs/treatment (half barrows and half gilts) were fasted overnight, electrically stunned (250 V and 0.5A for 5~6s), sacrificed (Wu, Liao, He, Feng, et al., 2015; Wu, Liao, He, Ren, et al., 2015), and then slaughtered. Samples of the feces and venous blood were collected from six randomly selected pigs per pen on d 35 of the nursery period. Venous blood samples were collected from all pigs in heparinized trace mineral-free Vacutainer (Becton Dickinson and Company, Franklin Lakes, NJ) tubes to determine serum biochemistry, amino acid (AA) profile and immunoglobulin levels (Liao et al., 2017). Serum was separated and stored as previously described (Liao et al., 2017), and the liver, spleen, kidneys and heart were removed, collected, and weighed as previously described (Liao et al., 2017; Wu, Liao, He, Feng, et al., 2015; Wu, Liao, He, Ren, et al., 2015).

2.4. Measurement of serum biochemistry, free AA profile, and immunoglobulin levels

The levels of albumin (ALB), glucose (GLU), creatinine (CREA), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), globulin (GLB), total cholesterol (TC), total proteins (TPs), urea (UREA), direct-acting bilirubin (D-BIL), indirect bilirubin (T-BIL), uric acid (UA), lactate dehydrogenase (LDH) and creatine kinase (CK) were determined using a Biochemical Analytical Instrument (Beckman CX_4 Chemistry Analyzer, Beckman Coulter, Brea, CA) and commercial kits (Nanjing Jiangcheng Biotechnology Institute, Jiangsu, China) as described previously (Duan et al., 2014; Liao et al., 2017; Wu, Liao, He, Feng, et al., 2015; Wu, Liao, He, Ren, et al., 2015).

The serum free AA profile was determined for three treatment of nursery pigs as described previously (Duan et al., 2014; Liao et al., 2017; Wu, Liao, He, Feng, et al., 2015; Wu, Liao, He, Ren, et al., 2015).

The levels of immunoglobulin G (IgG), immunoglobulin A (IgA), immunoglobulin M (IgM), and Complement C_3 (C_3) were determined using commercial ELISA kits (Cusabio Biotech Co., Ltd., Hubei, China) (Liao et al., 2017).

2.5. Chemical analysis of mineral levels

The mineral concentrations in the serum, feces, liver, longissimus dorsi (LD), spleen, and kidney were measured by inductively coupled plasma optical emission spectrometry (ICP-OES) (Agilent 7700, Agilent, Santa Clara, CA, USA) in nursery pigs in the three treatments as described previously (Liao et al., 2017; Xing, Hao, Liu, Xu, & Kuang, 2014).

2.6. Statistical analysis

The data were subjected to analysis of variance using the SAS software programme (Version 8.2; SAS Inst. Inc., Cary, NC) followed by Duncan's multiple comparison test

Table 2. The effects of dietary supplementation with NCG-Cu and copper sulfate on the growth performance of nursery pigs ($n = 6$).

Parameter	Dietary treatment			SEM ±	*P* value
	Control[1]	650 g/t Cu[2]	320 g/t NCG-Cu[3]		
Initial BW, kg	10.26	10.38	10.27	0.137	0.845
Final BW, kg	28.15[a]	29.76[a]	30.78[a]	0.317	0.034
ADG, g/d	511.14[a]	553.71[a]	586.00[a]	37.492	0.002
ADFI, g/d	783.55[a]	815.21[a]	828.72[a]	22.547	0.012
F/G	1.53[a]	1.47[a]	1.41[a]	0.019	0.004

[a]Means within a row without a common superscripted letter are significantly different ($P < 0.05$). The experiment lasted 35 d.
[1]Control = basal diet (Cu^{2+} element concentration is 10.24 mg/kg).
[2]650 g/t Cu = basal diet + 650 g/t copper sulfate (Cu^{2+} element concentration is 160 mg/kg).
[3]320 g/t NCG-Cu = basal diet + 320 g/kg cupreous N-carbamylglutamate chelate diet (Cu^{2+} element concentration is 80 mg/kg). NCG-Cu = cupreous N-carbamylglutamate chelate; BW = body weight; ADG = average daily gain; ADFI = average daily feed intake; F/G = the feed to gain ratio.

(Liao et al., 2017; Wu, Liao, He, Feng, et al., 2015; Wu, Liao, He, Ren, et al., 2015). The results were regarded as statistically significance at $P < 0.05$.

3. Results

3.1. Growth performance and diarrhea rate

The growth performance is shown in Table 2. The nursery pigs fed the 320 g/t NCG-Cu diet showed significant increases ($P < 0.05$) in final BW, ADFI, and F/G compared to those fed the 650 g/t Cu diet, and there were no significant differences between the 650 g/t Cu and control diets. Nursery pigs fed the 320 g/t NCG-Cu diet showed the highest ($P < 0.01$) ADG content among the three diets. Overall, pigs fed the 320 g/t NCG-Cu diet exhibited a significantly increased growth rate and feed conversion efficiency compared to those fed the 650 g/t Cu diet, while the growth performance of pigs fed the control diet showed an evidently decreasing trend. Pig growth performance was affected by the source or amount of Cu during the nursery period.

The diarrhea rate is shown in Table 3. The nursery pigs fed the 320 g/t NCG-Cu diet had a lower ($P < 0.05$) diarrhea rate than those fed the 650 g/t Cu diet, while there were no significant differences between the 650 g/t Cu and control diets. The pig diarrhea rate was affected by the source or amount of Cu during the nursery period.

Table 3. The effects of dietary supplementation with NCG-Cu and copper sulfate on the diarrhea rate in nursery pigs ($n = 6$).

Parameter	Dietary treatment			SEM ±	*P* value
	Control[1]	650 g/t Cu[2]	320 g/t NCG-Cu[3]		
Diarrhea rate (%)	4.10[a]	5.20[a]	3.92[a]	0.377	0.028

[a]Means within a row without a common superscripted letter are significantly different ($P < 0.05$). The experiment lasted 35 d. NCG-Cu = cupreous N-carbamylglutamate chelate.
[1]Control = basal diet (Cu^{2+} element concentration is 10.24 mg/kg).
[2]650 g/t Cu = basal diet + 650 g/t copper sulfate (Cu^{2+} element concentration is 160 mg/kg).
[3]320 g/t NCG-Cu = basal diet + 320 g/kg cupreous N-carbamylglutamate chelate diet (Cu^{2+} element concentration is 80 mg/kg). The diarrhea rate was calculated as follows: {piglets with diarrhea [*n*] / (total piglets in the experiment [*n*] × duration of the experiment [d])} × 100%.

Table 4. The effects of dietary supplementation with NCG-Cu and copper sulfate on the relative organ weights (g/kg BW) in nursery pigs ($n = 6$).

Organ	Dietary treatment			SEM ±	*P* value
	Control[1]	650 g/t Cu[2]	320 g/t NCG-Cu[3]		
Heart	4.67	5.13	4.54	0.217	0.628
Liver	22.47	27.04	24.43	2.325	0.299
Spleen	1.86	2.08	1.70	0.344	0.521
Kidney	4.95[a]	6.01[a]	4.92[a]	0.199	0.040

[a]Means within a row without a common superscripted letter are significantly different ($P < 0.05$). NCG-Cu = cupreous N-carbamylglutamate chelate. The experiment lasted 35 d.
[1]Control = basal diet (Cu^{2+} element concentration is 10.24 mg/kg).
[2]650 g/t Cu = basal diet + 650 g/t copper sulfate (Cu^{2+} element concentration is 160 mg/kg).
[3]320 g/t NCG-Cu = basal diet + 320 g/kg cupreous N-carbamylglutamate chelate diet (Cu^{2+} element concentration is 80 mg/kg).

3.2. Relative organ weights

Relative organ weights are shown in Table 4. The nursery pigs fed the 320 g/t NCG-Cu diet had lower ($P < 0.05$) relative kidney weights than those fed the 650 g/t Cu diet, and there were no significant differences between the 320 g/t Cu and control diets. No significant differences were observed among the three groups with respect to the heart, liver, or spleen weights ($P > 0.05$).

3.3. Serum biochemical profile and free AA and immunoglobulin levels

The serum biochemical profiles of the nursery pigs are listed in Table 5. No effects ($P > 0.05$) of the three diets were detected on the levels of GLU, GLB, TC, Urea, D-BIL,

Table 5. Serum biochemical parameters of nursery pigs fed diets containing NCG-Cu and copper sulfate ($n = 6$).

Parameter	Dietary treatment			SEM ±	*P* value
	Control[1]	650 g/t Cu[2]	320 g/t NCG-Cu[3]		
ALB (g/L)	42.23[a]	47.03[a]	40.13[a]	1.057	0.005
GLU (mmol/L)	7.06	7.98	7.34	0.197	0.446
CREA (mmol/L)	135.22[a]	146.47[a]	135.12[a]	3.890	0.043
ALP (U/L)	256.17[a]	383.55[a]	302.78[a]	32.510	0.028
ALT (U/L)	49.90[a]	68.08[a]	57.67[a]	5.930	0.027
AST (U/L)	52.88[a]	63.90[a]	57.27[a]	6.673	0.056
GLB(g/L)	13.42	15.48	14.55	0.990	0.387
TC (mmol/L)	2.50	2.93	2.81	0.177	0.084
TP (g/L)	55.65[a]	60.58[a]	55.62[a]	1.150	0.018
UREA (mmol/L)	3.19	4.07	3.01	0.383	0.161
D-BIL (μmol/L)	4.78	4.66	4.44	0.593	0.924
T-BIL (μmol/L)	4.27	4.37	4.02	2.150	0.940
UA (μmol/L)	0.00[a]	0.56[a]	0.11[a]	0.560	0.041
LDH (U/L)	664.63[a]	714.11[a]	643.34[a]	32.547	0.037
CK (U/L)	1951.05	1867.12	1823.43	453.150	0.081

[a]Means within a row without a common superscripted letter are significantly different ($P < 0.05$). The experiment lasted 35 d. NCG-Cu = cupreous N-carbamylglutamate chelate.
[1]Control = basal diet (Cu^{2+} element concentration is 10.24 mg/kg).
[2]650 g/t Cu = basal diet + 650 g/t copper sulfate (Cu^{2+} element concentration is 160 mg/kg).
[3]320 g/t NCG-Cu = basal diet + 320 g/kg cupreous N- carbamylglutamate chelate diet (Cu^{2+} element concentration is 80 mg/kg). ALB = albumin, GLU = glucose, CREA = creatinine, ALP = alkaline phosphatase, ALT = alanine aminotransferase, AST = aspartate aminotransferase, GLB = globulin, TC = total cholesterol, TPs = total proteins, UREA = urea, D-BIL = direct-acting-bilirubin, T-BIL = indirect bilirubin, UA = uric acid, LDH = lactate dehydrogenase, CK = creatine kinase.

T-BIL, and CK. The nursery pigs fed the 320 g/t NCG-Cu diet had lower ($P < 0.05$) levels of CREA, ALT, AST, TP, UA, and LDH than those fed the 650 g/t Cu diet, and there were no significant differences between the 320 g/t Cu and control diets. The ALB level in the 650 g/t Cu group was the highest ($P < 0.01$) among the three diets, and there were no significant differences between the 650 g/t Cu and control diets. Moreover, compared with the control, the 650 g/t Cu group displayed the highest ($P < 0.01$) ALP levels, while there were no significant differences between the 650 g/t Cu and 320 g/t Cu diets.

The serum-free AA profile results for the nursery pigs are listed in Table 6, and the levels of the six types of serum AA, including L-anserine, L-carnosine, L-cystine, Hydroxy-L-proline, O-Phospho-L-serine, and L-valine, were strongly affected by the three diets ($P < 0.05$).The nursery pigs fed the 320 g/t NCG-Cu diet had higher ($P < 0.05$) levels

Table 6. Serum free AA parameters of nursery pigs fed diets containing NCG-Cu and copper sulfate (*n* = 6).

	Dietary treatment				
Parameter	Control[1]	650 g/t Cu[2]	320 g/t NCG-Cu[3]	SEM ±	*P* value
L-1-Methylhistidine	0.00	0.00	0.00	0.000	None
L-3-Methylhistidine	0.79	0.83	0.84	0.066	0.901
L-alpha-Aminoadipic Acid	8.57	6.95	10.50	1.237	0.207
DL-α-Amino-n-butyric Acid	1.59	1.42	1.64	0.710	0.828
L-Alanine	53.18	57.90	47.77	6.430	0.571
L-Anserine	0.00[a]	0.64[a]	0.00[a]	0.097	0.023
L-Arginine	23.22	25.75	28.76	9.380	0.474
L-Aspartic Acid	3.38	3.73	3.05	1.180	0.532
DL-β-Aminoisobutyric Acid	0.00	0.00	0.00	0.000	None
β-Alanine	2.35	2.33	2.51	0.701	0.870
L-Carnosine	4.02[a]	4.24[a]	5.60[a]	1.126	0.048
L-Citrulline	11.39	12.70	11.51	4.830	0.548
L-Cystathionine	1.71	1.75	1.77	0.510	0.966
L-Cystine	0.90[a]	4.13[a]	1.84[a]	1.180	<0.0001
Ethanolamine	0.47	0.50	0.58	0.250	0.655
γ-Aminobutyric Acid	0.05	0.21	0.06	1.407	0.225
L-Glutamic Acid	37.45	41.94	38.16	7.167	0.837
Glycine	88.25	95.35	83.75	12.841	0.210
L-Histidine	5.37	4.87	5.33	2.220	0.884
DL-plus allo-δ-Hydroxylysine	0.53	1.63	0.90	1.190	0.343
Hydroxy-L-proline	15.14[a]	18.59[a]	17.32[a]	2.790	0.042
L-Isoleucine	13.68	15.43	16.39	2.890	0.175
L-Leucin	19.07	22.01	22.43	3.817	0.162
L-Lysine	38.36	41.17	35.91	5.757	0.831
L-Methionine	9.25	9.86	7.28	4.401	0.483
L-Ornithine	13.93	16.00	14.43	4.480	0.617
O-Phosphoethanolamine	0.00	0.00	0.00	0.000	None
L-Phenylalanine	10.56	10.76	10.07	2.350	0.830
L-Proline	29.34	31.95	27.57	6.510	0.396
O-Phospho-L-serine	6.34[a]	4.29[a]	4.35[a]	1.540	0.043
Sarcosine	3.86	3.70	3.97	3.430	0.458
L-Serine	18.58	21.59	17.23	4.740	0.189
Taurine	12.36	12.79	13.43	3.826	0.861
L-Threonine	19.86	28.60	27.44	10.090	0.202
L-Tyrosine	13.27	19.11	17.54	5.410	0.133
Urea	101.26[a]	95.96[a]	129.15[a]	42.720	0.267
L-Valine	18.65[a]	24.49[a]	29.52[a]	7.920	0.040

[a]Means within a row without a common superscripted letter are significantly different ($P < 0.05$). The experiment lasted 35 d. NCG-Cu = cupreous N-carbamylglutamate chelate.
[1]Control = basal diet (Cu^{2+} element concentration is 10.24 mg/kg).
[2]650 g/t Cu = basal diet + 650 g/t copper sulfate (Cu^{2+} element concentration is 160 mg/kg).
[3]320 g/t NCG-Cu = basal diet + 320 g/kg cupreous N- carbamylglutamate chelate diet (Cu^{2+} element concentration is 80 mg/kg).

Table 7. Serum immune parameters of nursery pigs fed with diets containing NCG-Cu and copper sulfate ($n = 6$).

Parameter	Dietary treatment			SEM ±	*P* value
	Control[1]	650 g/t Cu[2]	320 g/t NCG-Cu[3]		
IgG (g/L)	1.08[a]	1.39[a]	1.60[a]	0.507	0.022
IgA (g/L)	1.23[a]	1.63[a]	2.15[a]	1.422	0.023
IgM (g/L)	0.49[a]	0.30[a]	0.53[a]	0.117	0.005
C_3 (g/L)	0.15[a]	0.14[a]	0.16[a]	0.638	0.223

[a]Means within a row without a common superscripted letter are significantly different ($P < 0.05$). The experiment lasted 35 d. NCG-Cu = cupreous N-carbamylglutamate chelate.
[1]Control = basal diet (Cu^{2+} element concentration is 10.24 mg/kg).
[2]650 g/t Cu = basal diet + 650 g/t copper sulfate (Cu^{2+} element concentration is 160 mg/kg).
[3]320 g/t NCG-Cu = basal diet + 320 g/kg cupreous N-carbamylglutamate chelate diet (Cu^{2+} element concentration is 80 mg/kg). IgG = immunoglobulin G, IgA = immunoglobulin A, IgM = immunoglobulin M, C_3 = Complement C_3.

of L-carnosine, O-Phospho-L-serine, and L-valinethan those fed the control diet, but there were no significant differences between 320 g/t Cu and 650 g/t Cu diets except in the level of O-Phospho-L-serine. The nursery pigs fed the 650 g/t NCG-Cu diet had higher ($P <$ 0.05) levels of L-anserine, L-cystine, and Hydroxy-L- prolinethan those fed the control diet, and there were significant differences between the 320 g/t Cu and 650 g/t Cu diets, except for the level of Hydroxy- L-proline. Apart from those mentioned above, other AAs remained unaffected by the three diet treatments ($P > 0.05$).

The serum immune levels of the nursery pigs are listed in Table 7. The levels of IgG, IgA, and IgM in the serum were strongly affected by the three diets ($P < 0.05$), but the levels of C_3 did not differ significantly among the three groups ($P > 0.05$). The nursery pigs fed the 320 g/t NCG-Cu diet had the highest ($P < 0.05$) levels of IgG, IgA, and IgM than those fed the control diet, while there were significant differences between the 320 g/t Cu and 650 g/t Cu diets, except for the level of IgG.

3.4. Mineral concentrations in the feed, serum, fecal, organs, and LD

The mineral concentrations in the three feeds are listed in Table 8. Ten types of minerals were analyzed, among which the levels of Cu were 10.24 mg/kg,

Table 8. Analysis of the innate micromineral concentration of the basal diets, NCG-Cu and copper sulfate.

Parameter	Dietary treatment		
	Control[1]	650 g/t Cu[2]	320 g/t NCG-Cu[3]
P (mg/Kg)	3155.00	932.60	959.20
Mg (mg/Kg)	816.50	817.03	788.24
Ca (mg/Kg)	7813.50	7554.80	7357.40
Cd (mg/Kg)	0.33	0.11	0.20
Cu (mg/Kg)	10.24	308.17	272.72
Fe (mg/Kg)	396.98	407.23	374.50
Mn (mg/Kg)	82.57	68.47	85.20
Ni (mg/Kg)	2.07	2.14	2.12
Pb (mg/Kg)	1.79	0.99	0.94
Zn (mg/Kg)	1777.27	1735.82	1726.95

The experiment lasted 35 d. NCG-Cu = cupreous N-carbamylglutamate chelate.
[1]Control = basal diet (Cu^{2+} element concentration is 10.24 mg/kg).
[2]650 g/t Cu = basal diet + 650 g/t copper sulfate (Cu^{2+} element concentration is 160 mg/kg).
[3]320 g/t NCG-Cu = basal diet + 320 g/kg cupreous N-carbamylglutamate chelate diet (Cu^{2+} element concentration is 80 mg/kg). Each diet was analyzed in duplicate.

Table 9. Analyzed micromineral concentration of serum, feces and different organs in nursery pigs fed with diets containing NCG-Cu and copper sulfate ($n = 6$).

Parameter	Dietary treatment			SEM ±	*P* value
	Control[1]	650 g/t Cu[2]	320 g/t NCG-Cu[3]		
Serum					
Ca (mmol/L)	2.79[a]	2.77[a]	2.96[a]	0.317	0.550
P (mmol/L)	2.43[a]	2.50[a]	2.60[a]	1.567	0.176
Cu (μmol/L)	9.22[a]	18.31[a]	10.01[a]	17.452	0.026
Zn (μmol/L)	105.14[a]	91.21[a]	102.23[a]	41.891	0.024
Fe (μmol/L)	60.12[a]	64.71[a]	74.16[a]	19.062	0.006
Fecal					
Cu (mg/Kg)	855.43[a]	1531.41[a]	1005.26[a]	181.751	0.018
Zn (mg/Kg)	2701.12[a]	3523.10[a]	2835.41[a]	278.167	0.036
Fe (mg/Kg)	2036.55[a]	2634.57[a]	1840.53[a]	670.947	0.334
Liver					
Cr (mg/Kg)	7.93[a]	9.92[a]	9.61[a]	2.840	0.323
Mg (mg/Kg)	8.14[a]	10.07[a]	9.79[a]	2.941	0.351
Ca (mg/Kg)	813.62[a]	761.14[a]	788.48[a]	177.027	0.082
Cd (mg/Kg)	5.51[a]	6.68[a]	5.67[a]	3.481	0.811
Cu (mg/Kg)	1533.81[a]	1946.65[a]	1250.81[a]	157.213	0.047
Fe (mg/Kg)	1571.72[a]	1948.80[a]	1281.65[a]	716.750	0.035
Mn (mg/Kg)	897.95[a]	844.62[a]	868.25[a]	56.850	0.127
Ni (mg/Kg)	1454.72[a]	1953.45[a]	1260.01[a]	636.351	0.094
Pb (mg/Kg)	1511.81[a]	1940.51[a]	1244.06[a]	613.327	0.142
Zn (mg/Kg)	163.04[a]	287.13[a]	174.20[a]	84.176	<0.0001
LD					
Cr (mg/Kg)	9.48[a]	11.98[a]	11.49[a]	3.780	0.341
Mg (mg/Kg)	1135.35[a]	1191.93[a]	1216.06[a]	158.962	0.618
Ca (mg/Kg)	2328.62[a]	2557.28[a]	3090.04[a]	352.293	0.383
Cd (mg/Kg)	0.27[a]	0.21[a]	0.24[a]	0.254	0.912
Cu (mg/Kg)	31.00[a]	41.92[a]	41.15[a]	11.781	0.095
Fe (mg/Kg)	124.02[a]	193.03[a]	134.85[a]	67.951	0.082
Mn (mg/Kg)	4.31[a]	5.47[a]	5.40[a]	1.652	0.289
Ni (mg/Kg)	3.37[a]	4.66[a]	3.07[a]	1.061	0.037
Pb (mg/Kg)	3.41[a]	2.10[a]	2.75[a]	3.768	0.747
Zn (mg/Kg)	123.73[a]	122.61[a]	176.48[a]	66.471	0.203
Spleen					
Cr (mg/Kg)	16.12[a]	11.36[a]	11.26[a]	4.686	0.169
Mg (mg/Kg)	1055.35[a]	1065.66[a]	826.56[a]	375.153	0.390
Ca (mg/Kg)	2506.86[a]	2463.02[a]	2463.10[a]	768.381	0.994
Cd (mg/Kg)	0.24[a]	0.20[a]	0.29[a]	0.218	0.833
Cu (mg/Kg)	29.86[a]	48.81[a]	30.19[a]	5.574	0.043
Fe (mg/Kg)	785.73[a]	571.64[a]	453.33[a]	59.795	0.005
Mn (mg/Kg)	9.41[a]	5.20[a]	7.98[a]	3.350	0.038
Ni (mg/Kg)	3.77[a]	2.13[a]	1.45[a]	2.175	0.232
Pb (mg/Kg)	7.30[a]	0.97[a]	3.48[a]	4.881	0.145
Zn (mg/Kg)	160.58[a]	166.17[a]	117.36[a]	37.358	0.205
Kidney					
Cr (mg/Kg)	11.27[a]	13.12[a]	19.85[a]	4.635	0.008
Mg (mg/Kg)	930.85[a]	991.15[a]	968.32[a]	74.628	0.239
Ca (mg/Kg)	2389.30[a]	2471.16[a]	2197.23[a]	551.028	0.814
Cd (mg/Kg)	0.51[a]	2.03[a]	2.19[a]	0.264	0.001
Cu (mg/Kg)	109.55[a]	1114.56[a]	609.90[a]	273.690	0.003
Fe (mg/Kg)	327.93[a]	199.64[a]	279.66[a]	96.897	0.068
Mn (mg/Kg)	11.65[a]	12.31[a]	13.49[a]	4.091	0.369
Ni (mg/Kg)	4.66[a]	1.46[a]	4.02[a]	3.674	0.199
Pb (mg/Kg)	1.57[a]	7.25[a]	4.55[a]	7.775	0.509
Zn (mg/Kg)	437.40[a]	540.06[a]	535.67[a]	180.015	0.491

[a]Means within a row without a common superscripted letter are significantly different ($P < 0.05$). NCG-Cu = cupreous N-carbamylglutamate chelate. LD = longissimus dorsi. The experiment lasted 35 d.
[1]Control = basal diet (Cu^{2+} element concentration is 10.24 mg/kg).
[2]650 g/t Cu = basal diet + 650 g/t copper sulfate (Cu^{2+} element concentration is 160 mg/kg).
[3]320 g/t NCG-Cu = basal diet + 320 g/kg cupreous N-carbamylglutamate chelate diet (Cu^{2+} element concentration is 80 mg/kg). Each sample was analyzed in three times.

308.17 mg/kg and 272.72 mg/kg in the control, 650 g/t Cu, and 320 g/t NCG-Cu diets, respectively.

The serum, fecal, organ (liver, spleen, and kidney), and LD mineral levels are listed in Table 9. Serum Cu was affected by both the source and amount of supplementation, and its levels were greater ($P < 0.05$) in the two Cu-supplemented diets than the control diet. There were no significant differences between the 320 g/t Cu and control diets. Pigs fed the control diet had greater ($P < 0.05$) serum Zn than those given the 650 g/t Cu diets, but the level did not differ from those fed the 320 g/t Cu diets. Serum Fe was highest ($P < 0.05$) with the 320 g/t Cu diet compared with the control and 650 g/t Cu diets but did not differ from those fed the control and 650 g/t Cu diets.

Fecal Cu levels increased ($P < 0.05$) as the dietary levels of Cu increased (Table 9). Specifically, fecal Cu levels were increased when pigs were fed the 650 g/t Cu diet compared with those fed the 320 g/t Cu diet ($P < 0.05$). Pigs supplemented with 650 g/t Cu had greater ($P < 0.05$) fecal Zn than those fed the control and 320 g/t Cu diets, but those fed the control and 320 g/t Cu diets showed no difference.

Pigs fed 650 g/t Cu had greater ($P < 0.05$) liver Cu and liver Fe than those fed 320 g/t Cu diet, but the levels did not differ between those fed the control and 320 g/t Cu diets. Compared with those fed the control diet, pigs fed 650 g/t Cu and 320 g/t Cu had greater ($P < 0.01$) liver Zn levels. Liver Zn was also greater ($P < 0.01$) with the 650 g/t Cu diet than the 320 g/t Cu diet.

The LD Ni levels were higher ($P < 0.05$) with the 650 g/t Cu diets compared with the 320 g/t Cu and control diets but did not differ between the 320 g/t Cu and control diets.

The spleen Cu level was higher ($P < 0.05$) with the650 g/t Cu diet compared with the 320 g/t Cu and control diets, but it did not differ between the 320 g/t Cu and control diets. Spleen Fe was also greater ($P < 0.01$) with the control than the 650 g/t Cu diets but did not differ between the 650 g/t Cu and 320 g/t Cu diets. Spleen Mn was also greater ($P < 0.05$) in the control than the 650 g/t Cu diets but did not differ between the 320 g/t Cu and control diets.

Kidney Cr was higher ($P < 0.05$) with the 320 g/t Cu diets compared with the 650 g/t Cu and control diets, but it did not differ between the 650 g/t Cu and control diets. Kidney Cd was also lowest ($P < 0.01$) with the control than the 320 g/t Cu and 650 g/t Cu diets, but it did not differ between the 650 g/t Cu and 320 g/t Cu diets. Compared with those fed the control diet, pigs fed 650 g/t Cu and 320 g/t Cu had greater ($P < 0.01$) kidney Cu levels. Kidney Cu was also greater ($P < 0.01$) with 650 g/t Cu than 320 g/t Cu.

4. Discussion

Numerous studies have shown the effects of growth promotion and reduced diarrhea in animals fed diets supplemented with Cu as either copper sulfate or organic (Beames & Lloyd, 1965; Hasman et al., 2006; Hill et al., 2000; Liao et al., 2017; Xing et al., 2014; Yuan et al., 2015). In the current study, the growth performance of pigs fed 320 g/t NCG-Cu (organic trace minerals) significantly increased in terms of growth rate and feed conversion efficiency compared to those fed the 650 g/t Cu diet (inorganic trace minerals) (Tables 2 and 3). These observations are consistent with those of previous studies, in which the growth of piglets was stimulated, and the diarrhea rate was reduced (Armstrong et al., 2004; Fry et al., 2012; Gonzales-Eguia et al., 2009; Huang et al., 2015; Liao et al., 2017;

Xing et al., 2014). Some studies have shown that piglets fed diets containing increased concentrations of an organic trace mineral premix had better growth performance than pigs fed inorganic sources of trace minerals (Coffey et al., 1994; Zhou, Kornegay, van Laar, et al., 1994), but a previously study also showed that the growth rate was only stimulated in the grower stage by increasing feed intake but not during the finishing period (Smits & Henman, 2000). However, some studies have shown that while pigs fed diets supplemented with organic Cu exhibited similar levels of growth as those fed diets supplemented with inorganic Cu, organic or chelate Cu can substantially reduce the amount of fecal Cu excretion (Acda & Chae, 2002; Lee, Choi, Chae, Acda, & Han, 2001; Smits & Henman, 2000). These discrepancies may be due to differences in host-related factors such as age and the species of animal, sex, stage of growth, pregnancy, lactation, nutritional status, disease, gastrointestinal (GIT) secretions and microflora as well as GIT transit time (Acda & Chae, 2002).

The relative kidney weights did not differ between the pigs in the two Cu-supplementation treatments in the current study (Table 4), but other investigators have found an increase in the relative organ weights of both male and female rats exposed to copper-deficient diets (Allen, Hassel, & Lei, 1982; Koller, Mulhern, Frankel, Steven, & Williams, 1987) or decreased relative kidney weights in pigs fed 650 g/t Cu and 640 g/t NCG-Cu diets (Liao et al., 2017). These discrepancies may be due to differences in the sources, age, dosage, or purity of the Cu used in the different experiments (Acda & Chae, 2002; Liao et al., 2017).

Serum biochemistry parameters are sensitive serological indicators of liver and kidney toxicity under different physiological conditions (Duan et al., 2014; Liao et al., 2017; Wu, Liao, He, Feng, et al., 2015; Wu, Liao, He, Ren, et al., 2015). In the current study, the pigs fed the 320 g/t NCG-Cu diet had lower ($P < 0.05$) levels of CREA, ALT, AST, TP, UA, LDH, and ALB than those fed the 650 g/t Cu diet, but there were no significant differences between pigs fed the 320 g/t Cu and control diets. The increasing activities of CREA, ALT, AST, TP, UA, LDH, and ALB showed the effects of the two different Cu sources on renal and hepatic functions, and this result is supported by previous research indicating that dietary supplementation with copper (at concentrations ranging from 100 to 1000 mg Cu/kg diet) has a significant effect on ALT, AST, TP, UA in quail, hens, and weanling pigs (Almansour, 2006; Güçlü et al., 2008; Liao et al., 2017). However, this is not consistent with the results of our previous studies showing that increased CREA, ALB, and LDH in weanling pigs after feeding with 650 g/t Cu and 640 g/t NCG-Cu diets (Liao et al., 2017). This difference between animal species and the source of Cu is possibly due to liver damage from Cu exposure.

Serum free AA concentrations reflect the physiological conditions and nutritional state in animals (Liao et al., 2017), and it has been clearly demonstrated that citrulline and arginine levels increase in pigs fed a single NCG-contaminated diet (Wu et al., 2010). A previous study showed that the concentrations of DL-α-amino-n-butyric acid, L-alanine, L-cystathionine, L-lysine, L-ornithine, L-phenylalanine, and L-tyrosine with 650 g/t Cu and 640 g/t NCG-Cu diets were significantly different from those with a control diet (Liao et al., 2017). In the present study, the levels of L-anserine, L-carnosine, L-cystine, Hydroxy-L-proline, O-Phospho-L-serine, and L-valine were strongly affected by the three diets ($P < 0.05$), indicating that different amounts and sources of dietary Cu led to the increased availability of free AA (especially L-anserine, L-carnosine, L-cystine, Hydroxy-L-proline, O-Phospho-L-serine, and L-valine) in the serum and suggesting differences in the absorption of the three diets.

Different amounts and sources of Cu can promote the immune response (Creech et al., 2004; Güçlü et al., 2008; Herich, 2017; Koller et al., 1987; Kornegay, van Heugten, Lindemann, & Blodgett, 1989; Liao et al., 2017). In a previous study, two concentrations (67 and 134 mg Cu/kg) of $CuSO_4$ and Cu-methionine (Cu-Met) did not affect immune indicators, including IgG and lymphocytes (Huang et al., 2010). Other previous studies have shown that 20 mg Cu/kg of $CuSO_4$ and Cu-Met can decrease milk production and increase the concentration of phase IgG but not affect somatic the cell count in dairy cows (Paik, 2001). In the present study, the immune-stimulatory properties of the 320 g/t NCG-Cu diet may be superior to that of the 650 g/t $CuSO_4$ diet, except for IgG. The level of Cu can affect T and B cells, neutrophils and macrophages as well as impair immune function (Liao et al., 2017; Punyokun, Hongprayoon, Srisapoome, & Sirinarumitr, 2013; Qiao et al., 2017). These results suggest that dietary supplementation with 320 g/t NCG-Cu may induce an immune response in piglets by modulating immunoglobulin levels.

Serum Cu levels are affected by the intake concentration of dietary Cu. In the present study, serum Cu levels were affected by the source and amount of Cu and were greater ($P < 0.05$) with the two Cu-supplemented diets than the control. These data are not consistent with our previous results that plasma Cu levels in weanling piglets were not affected by dietary supplementation with 650 g/t Cu and the 640 g/t NCG-Cu (Liao et al., 2017). However, dietary supplementation with 225 mg Cu/kg of $CuSO_4$ can increase the concentrations of plasma Cu in nursery piglets (Armstrong, Williams, Spears, & Schiffman, 2000). Previous studies have shown that dietary supplementation with Cu from copper sulfate and a copper lysine complex at concentrations of 100, 150, and 200 mg/kg Cu can increase the concentrations of plasma Cu in weanling pigs. These discrepancies may be due to differences in the sources and dosage of the Cu as well as the age of the animals used in different experiments.

In the present study, supplementation with 320 g/t NCG-Cu did not significantly enhance tissue mineral concentrations, expect for the Cu levels in the liver and kidney, compared to the control. The liver Cu was high after birth, so young animals may have been at risk due to artificial feeding. Furthermore, there was a striking effect of age on the tissue Cu level due to Cu supplementation. Numerous studies have shown a large increase in the liver Cu level as well as an increase in the kidney Cu level in weanling piglets fed high-concentration Cu diets, which is consistent with the results of our present study (Cromwell, Stahly, & Monegue, 1989; Liao et al., 2017; Luo & Dove, 1996). Therefore, the results showed that when Cu is added to the diet at a high concentration, the distribution of Cu varies greatly among the different organs in young animals with more Cu being distributed in the liver and kidney, within the physiological range, according to the changes in the level of Cu supplementation.

Although pig performance improves with supplementation of high concentrations of Cu as $CuSO_4$, there are environmental concerns associated with high concentrations of Cu in manure (Fry et al., 2012; Huang et al., 2015; Liao et al., 2017). In the present study, nursery piglets fed the 320 g/t NCG-Cu diet showed decreased fecal excretion of Cu and Zn compared with piglets fed the 650 g/t Cu diet, which is consistent with the results of a previous study (Armstrong et al., 2004; Liao et al., 2017). Fecal excretion of Cu and Zn (in mg per day) is directly related to the quantity of Cu and Zn consumed (in mg per day) regardless of the source (Apgar et al., 1995; Apgar & Kornegay, 1996; Carlson et al., 2004; Cromwell et al., 1998). Decreasing the Zn and Cu in pig feces is important because accumulation of these minerals in soil can lead to toxicity in numerous plants, so it can potentially boost the sustainability of the swine industry in terms of environmental health (Liao et al., 2017).

5. Conclusions

In summary, the results of the study indicated that the growth- or immune- stimulatory effects of 320 g/t NCG-Cu are superior to those of 650 g/t Cu. Because of the reduction in fecal Cu concentrations, 320 g/t NCG-Cu (chelated) dietary Cu may provide an effective environmental alternative to 650 g/t Cu (inorganic) for nursery piglets, and Cu was also shown to have limited effects on tissue mineral distributions, except in the spleen and liver. This study contributes to further understanding the application of chelated Cu and inorganic Cu mineral diets as a nutrition strategy for swine and other young mammals to reduce dietary fecal excretion and thus Cu pollution in soil and water worldwide.

Disclosure statement

No potential conflicts of interest were reported by the authors.

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ORCID

Peng Liao http://orcid.org/0000-0001-5740-7272

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