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Dietary Chitosan-Cu Chelate Affects Growth Performance and Small Intestinal Morphology and Apoptosis in Weaned Piglets

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ABSTRACT

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The effects of dietary chitosan-copper chelate (CS-Cu) on growth performance, diarrhea, intestinal morphology and epithelial cell apoptosis in weaned piglets was investigated. One hundred and sixty Duroc × Landrace × Yorkshire weanling barrows with an average body weight of 7.75 kg were randomly assigned to one of the following dietary treatments: (1) control, (2) 100 mg Cu/kg diet from $CuSO_4$, (3) 100 mg Cu/kg diet from $CuSO_4$ mixed with chitosan ($CuSO_4$+CS), (4) 100 mg Cu/kg diet from CS-Cu. The feeding trial lasted for 30 days. The results showed that the pigs receiving a diet containing CS-Cu had higher average daily gain and lower diarrhea incidence than the pigs receiving dietary $CuSO_4$ and $CuSO_4$+CS. Villus height and the ratio of villus height/crypt depth in duodenum, jejunum, and ileum were higher and crypt depth was lower in CS-Cu treated pigs than in pigs fed dietary $CuSO_4$ or $CuSO_4$+CS. An apparent decrease of ileal epithelial cell apoptosis in pigs fed CS-Cu diet was found. The activities of antioxidant enzymes were higher in pigs fed dietary CS-Cu than in those fed other diets. The results indicated that dietary CS-Cu showed better biological and physiological function in improving small intestinal morphology and reducing diarrhea incidence.

Keywords: CS-Cu; villus morphology; daily gain; diarrhea incidence; ileal epithelial cell; antioxidant enzymes

Copper (Cu) can activate many enzymes and plays a crucial role in biochemical reactions and physiological regulations. It is essential for animal reproduction, bone development, and growth (Underwood and Suttle 1999). Some studies showed that dietary supplementation with various Cu sources could increase daily gain in swine (Cromwell et al. 1998; Hill et al. 2000). Its provision in sufficient amount in pig feeding is therefore indispensable to ensure good performance and to maintain animals in good health. Due to the generally demonstrated beneficial response on daily feed consumption and growth performance of weanling piglets, the supplementation of high Cu levels (200–250 mg/kg) to nursery pig is currently a common practice (Hill et al. 2000; Shelton et al. 2011). Usually, additional Cu is most efficacious for growth of weanling pigs at 200–250 mg/kg (Cromwell 2001). However, since as much as 90% of the Cu ingested by pigs is excreted in feces and urine, feeding high levels of Cu increases its emission and becomes a potential environmental threat (Kornegay and Verstegen 2001). The subsequent application of manure on soil may be toxic to plants and microorganisms (Aldrich et al. 2002).

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To resolve these problems, usage of Cu in the form of chelates, complexes or proteinates in animal diet as the alternatives to inorganic sources was considered. This is probably due to their higher biological activity and absorption, improving the use efficiency and reducing the feed supplement with the result of lower faecal excretion and environmental impact (Guo et al. 2001). Chitosan is derived from chitin, a linear polysaccharide. It is found in the exoskeleton of shellfish such as shrimp, lobster, or crabs. Chitosan-Cu (CS-Cu) is a chelate of Cu^{2+} with chitosan, and previous studies on the chelation of Cu^{2+} with chitosan were focused on its applications in metal ions separation or treatment of waste water (Mcafee et al. 2001). Our previous studies showed that Cu-loaded chitosan (containing 2% of Cu, not chitosan-Cu chelate) improved biological functions in rats (Han et al. 2010). Dietary chitosan-Zn (CS-Zn) chelate could improve morphology and mucosal antioxidant enzyme activity and immune function of intestine in weaned piglets (Han et al. 2014; Ma et al. 2014).

The objective of the present study was to investigate whether CS-Cu chelate could maintain growth performance, improve small intestinal morphology, and retard epithelial cell apoptosis of weanling pigs compared with $CuSO_4$ and $CuSO_4$ mixed with chitosan.

MATERIAL AND METHODS

All experimental procedures involving animal care and sampling were approved by the Zhejiang University Animal Care and Use Committee (Hangzhou, China).

Animals and experimental design. A total of 160 crossbred (Duroc × Landrace × Yorkshire) weanling barrows (28 ± 2 days of age) with an average body weight of 7.76 kg (± 0.23 kg) were randomly assigned to one of the following dietary treatments: (1) control (no supplemental Cu), (2) 100 mg supplemental Cu/kg diet from $CuSO_4$, (3) 100 mg supplemental Cu/kg diet from $CuSO_4$ mixed with chitosan ($CuSO_4$+CS, the content of chitosan was the same as that of CS-Cu), (4) 100 mg supplemental Cu/kg diet from CS-Cu. Chitosan-Cu, a Cu-chitosan chelate compound containing 15.7% of Cu, was provided by the Feed Science Institute of Zhejiang University (Hangzhou, China). Each treatment was replicated four times with ten pigs per replicate (i.e. pen). The pens had concrete floors, and each pen was equipped with a feeder and nipple drinker. All animals had *ad libitum* access to feed and water. The feeding trial lasted 30 days. All animals were fed a corn-soybean meal basal diet (control) (Table 1). The content of Cu in basal diet was 41.8 mg/kg, and all nutrients met or exceeded NRC (1998) recommendations for weanling piglets.

Feed intake was recorded daily per pen. Pigs were weighed individually at the start and end of the feeding trial, and average daily gain (ADG), average daily feed intake (ADFI), and feed to gain ratio (F/G) were calculated for each pen. The number of weaned piglets with diarrhea and its duration were observed and recorded during the trial. Diarrhea was defined as liquid consistency over a minimum of 2 consecutive days (Manner and Spieler 1997). The incidence of diarrhea (%) was calculated as the total number of diarrheal piglets during the period divided by the total number of piglets multiply duration of feeding trial.

Table 1. Dietary ingredient and nutrient contents of the basic diets (as-fed basis)[1]

Ingredients (%)		Chemical composition[3]	
Corn	64.00	digestible energy (MJ/kg)	13.6
Soybean meal	28.00	crude protein (%)	17.5
Whey power	2.50	ether extract (%)	3.9
Soybean oil	2.00	Ca (%)	0.95
Monocalcium phosphate	1.00	P (%)	0.68
Limestone	1.20	lysine (%)	1.52
Salt	0.30	methionine + cysteine (%)	0.78
Vitamin-mineral premix[2]	1.00	Cu (mg/kg)	41.8

[1]nutrient content of the diets was based on NRC (1998) recommendations

[2]Vitamin-mineral premix provided (per kg basic diet): Vitamin A 6000 IU, Vitamin D 1500 IU, Vitamin E 30 IU, Vitamin K 2.0 mg, Vitamin B_1 2.0 mg, Vitamin B_2 5.5 mg, Vitamin B_6 1.6 mg, biotin 0.25 mg, folic acid 1.25 mg, pantothenic acid 18.0 mg, niacin 20 mg, Fe (as $FeSO_4 \cdot 7H_2O$) 200 mg, Zn (as $ZnSO_4 \cdot 7H_2O$) 105 mg, Mn (as $MnSO_4 \cdot H_2O$) 30 mg, I (as ethylenediamine dihydroiodide) 0.3 mg, Se (as sodium selenite) 0.2 mg

[3]analyzed composition except for the digestible energy value

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Sample collection and preparation. At the end of the feeding trial, eight pigs (2 pigs per pen) from each dietary treatment were selected based on similar body weight and euthanized. Subsequently, the abdominal cavity was opened vertically and the samples of gastrointestine and liver were collected. Liver sample for the examination of antioxidant enzyme activity was shock-frozen in liquid nitrogen. All samples were stored at –70°C until required for analysis.

The duodenum, jejunum, and ileum segments for histologic evaluation were fixed with 10% formalin at 4°C. Subsequently, the samples were embedded in paraffin for hematoxylin and eosin staining. The histological processing and determination was carried out as described by Van Dijk et al (2001). Well-oriented crypt-villus units were selected for each intestinal cross-section (15 measurements for each sample). Villus height and crypt depth were examined via a light microscope (Olympus, Japan) and measurements were obtained using the Image Processing and Analysis System, Version 1 (Leica Imaging Systems Ltd., UK).

Immunohistochemistry and analysis methods. Ileum samples from each group which were sectioned at 5 μm on a rotary microtome were fixed in 10% formalin and then embedded paraffin wax for analyzing apoptosis of mucosa epithelial cells with a modified TUNEL method (Gavrieli et al. 1992). The number of TUNEL-stained epithelial cells was counted in 15–20 selected sections from each group. Sections were examined using a light microscope. A semi-quantitative procedure was used to count the number of epithelial cells undergoing apoptosis and then the apoptotic index was calculated. The apoptotic index for each field was determined as the number of positive TUNEL-stained epithelial cells divided by the total number of epithelial cells counted per field. The means and standard errors were obtained from these counts.

Chemical analysis. Liver samples were homogenized with vitreous homogenizer in ten volumes (w/v) of 0.25M sucrose, containing 20 mM Tris–HCl, pH 7.4, 0.1% peroxide-free Triton X-100, and centrifuged at 1000 *g* for 20 min at 4°C. Then, the supernatant was collected. Total superoxide dismutase (SOD) was measured with xanthine oxidase method (Pocker1984). One unit of SOD activity is defined as the amount of protein that inhibits the rate of nitrite reduction by 50%. Glutathione peroxidase (GPx) activity was determined by the method of DTNB colorimetry at 412 nm (Sazuka et al. 1989). Catalase (CAT) activity was measured according to the method of Beers and Sizer (1954), based on the decomposition of H_2O_2 per minute (Beers and Sizer 1954). Protein content of the homogenates was examined using the Quick Start Bradford Protein Assay (Bio-Rad, USA) according to the manufacturer's instructions. SOD, GPx, and CAT activities were expressed in terms of units/mg protein.

Statistical analysis. Data were analyzed by one-way ANOVA using the General Linear Mixed Models procedure of the SAS software (Statistical Analysis System, Version 6.12, 1996). A pen was used as the experimental unit for growth data, whereas an individual pig was used as the experimental unit for the remaining traits. Data were presented as means and standard errors of the means. Statistical significance was assessed using Tukey's HSD test. An alpha level of 0.05 was used to determine statistical significance of differences between treatments.

RESULTS

Performance. Effects of CS-Cu on growth performance and diarrhea incidence of weaned pigs are shown in Table 2. Dietary Cu enhanced ADG of pigs apparently. In days 1–14 or 1–30 post weaning, ADG increased in pigs fed the diets supplemented with CS-Cu compared with those fed other diets ($P < 0.05$), no difference was found between $CuSO_4$ and $CuSO_4$+CS treatments ($P > 0.05$). From day 1 to day 14 post weaning, there was no difference in F/G between all treatments ($P > 0.05$). However, from day 1 to day 30 post weaning, the pigs fed dietary CS-Cu had lower F/G than pigs fed other diets ($P = 0.14$). Moreover, no difference was found between $CuSO_4$ and $CuSO_4$+CS treatments ($P > 0.05$). The incidence of diarrhea decreased in pigs fed the diets supplemented with copper from either CS-Cu, $CuSO_4$+CS or $CuSO_4$ ($P < 0.01$) compared with control pigs. Moreover, the pigs fed dietary CS-Cu had lower diarrhea incidence than those receiving 100 mg/kg $CuSO_4$+CS or $CuSO_4$ ($P < 0.05$).

Liver antioxidant enzyme activities. Table 3 shows the activities of SOD, GPx, and CAT in liver. Compared with control, the SOD and GPx activities increased in other treatments ($P < 0.05$).

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Table 2. Growth performance and incidence of diarrhea of weaned piglets (n = 8)

Cu source (mg Cu/kg diet)	Control	$CuSO_4$	$CuSO_4$+CS	CS-Cu	SEM	P-value
	0	100	100	100		
Body weight (kg)						
Day 1	7.83	7.72	7.75	7.70	0.05	0.13
Day 14	12.15^b	12.42^b	12.51^b	13.44^a	0.27	< 0.01
Day 30	19.81^b	20.51	20.86	22.84^a	0.26	< 0.01
Average daily gain (g)						
Days 1–14	310^c	337^b	342^b	413^a	6	< 0.01
Days 15–30	512^c	541^b	558^b	631^a	9	< 0.01
Days 1–30	400^c	428^b	439^b	506^a	11	< 0.01
Feed : gain						
Days 1–14	1.68	1.66	1.66	1.55	0.07	0.31
Days 15–30	2.12^a	2.01^a	1.98^a	1.81^b	0.14	0.14
Days 1–30	2.08^a	1.98^a	1.94^a	1.76^b	0.13	0.14
Incidence of diarrhea[1] (%)	14.42^a	7.28^b	6.93^b	5.10^c	1.25	< 0.01

CS = chitosan, CS-Cu = chitosan-copper chelate, SEM = standard error of the means

[1]total number of diarrheal piglets over the period divided by the total number of piglets multiply duration of feeding trial

[a–c]data within the same row with different superscripts differ significantly (P < 0.05)

Moreover, the activities of SOD and GPx were higher in pigs fed 100 mg Cu/kg diet from CS-Cu than in those fed other Cu sources (P < 0.05). No difference was found between pigs fed dietary $CuSO_4$+CS and $CuSO_4$ (P > 0.05). Dietary Cu supplementation did not affect the activities of CAT in liver (P = 0.23).

Intestinal mucosal histology and ileal epithelial cells apoptosis. The villus height and crypt depth of small intestine and cell apoptosis of ileum epithelium are shown in Table 4 and Figure 1. Villus heights of duodenum, jejunum, and ileum were higher in pigs fed the diets supplemented with CS-Cu than in pigs fed other diets, the villus heights in pigs of $CuSO_4$ and $CuSO_4$+CS treatments were higher than of the control, no difference was found between $CuSO_4$ and $CuSO_4$+CS treatments. Whereas crypt depths of duodenum, jejunum, and ileum were lower in pigs fed Cu-supplemented diets than in control pigs (P < 0.05), and the crypt depths were lower in pigs fed CS-Cu than other Cu sources, there were no differences between $CuSO_4$ and $CuSO_4$+CS treatments. The ratio of villus height/crypt depth was greater in pigs fed Cu-supplemented diets than in control pigs (P < 0.05), and the ratio was greater in CS-Cu treatments than in $CuSO_4$ and $CuSO_4$+CS treatments However, there were no differences between the pigs fed 100 mg Cu/kg $CuSO_4$ or $CuSO_4$+CS diet (P > 0.05).

Table 3. Effect of CS-Cu on antioxidant enzymes activities in liver of weaned piglets (n = 8)

Cu source (mg Cu/kg diet)	Control	$CuSO_4$	$CuSO_4$+CS	CS-Cu	SEM	P-value
	0	100	100	100		
SOD (IU/mg protein)	302^c	320^b	324^b	380^a	13	< 0.01
GPx (IU/mg protein)	82^c	101^b	107^b	148^a	4.7	< 0.01
CAT (IU/mg protein)	68	71	71	76	2.9	0.23

CS = chitosan, CS-Cu = chitosan-copper chelate, SOD = superoxide dismutase, GPx = glutathione peroxidase, CAT = catalase, SEM = standard error of the means

[a–c]data within the same row with different superscripts differ significantly (P < 0.05)

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Table 4. Effect of CS-Cu on villus height and crypt depth of small intestine in weaned piglets (n = 8)

Cu source (mg Cu/kg diet)	Control	$CuSO_4$	$CuSO_4$+CS	CS-Cu	SEM	P-value
	0	100	100	100		
Duodenum (μm)						
Villus height	442[c]	508[b]	511[b]	551[a]	13	< 0.01
Crypt depth	359[a]	319[b]	312[b]	280[c]	14	< 0.01
Villus height/crypt depth	1.23[c]	1.61[b]	1.63[b]	1.94[a]	0.12	< 0.01
Jejunum (μm)						
Villus height	306[c]	419[b]	420[b]	464[a]	11	< 0.01
Crypt depth	233[a]	220[b]	218[b]	182[c]	8	< 0.01
Villus height/crypt depth	1.34[c]	1.92[b]	1.94[b]	2.51[a]	0.13	< 0.01
Ileum (μm)						
Villus height	304[c]	384[b]	389[b]	432[a]	11	< 0.01
Crypt depth	195[a]	178[b]	175[b]	142[c]	6	< 0.01
Villus height/crypt depth	1.64[c]	2.22[b]	2.24[b]	3.03[a]	0.12	< 0.01

CS = chitosan, CS-Cu=chitosan-copper chelate, SEM = standard error of the means

[a–c]data within the same row with different superscripts differ significantly (P < 0.05)

The apoptotic index of ileal epithelial cells is given in Figure 1. Microscopic examination showed that there were lots of TUNEL-stained ileal epithelial cells undergoing apoptosis in pigs fed control diet. Many stained ileal epithelial cells were also observed in pigs fed diets supplemented with 100 mg/kg Cu form or $CuSO_4$+CS. Relatively few stained ileal epithelial cells were found in pigs fed CS-Cu diets. Calculation of the apoptotic index from the quantification of TUNEL-stained cells showed an apparent decrease in ileal epithelial cells apoptosis in pigs fed Cu-supplemented diets (P < 0.05) (Figure 1). The apoptotic index of ileal epithelial cells in pigs fed 100 mg CS-Cu/kg diet was lower than in $CuSO_4$ or $CuSO_4$+CS treated pigs (P < 0.05). No difference was found between pigs fed dietary $CuSO_4$ and $CuSO_4$+CS (P > 0.05).

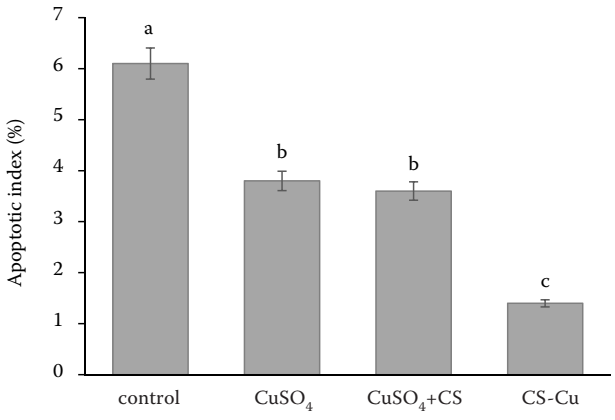

Figure 1. Effect of chitosan-copper chelate (CS-Cu) on apoptotic index of ileal epithelial cells in weaned piglets bars represent mean ± standard error of the mean, and bars not sharing a common letter are different (P < 0.05)

DISCUSSION

Performance. In the present study, dietary Cu improved growth rate and feed efficiency. The results are in line with previous studies in which supplementation of Cu as $CuSO_4$ at concentrations of 125 to 250 mg/kg stimulated growth and improved feed efficiency in weanling pigs (Cromwell et al. 1989; Armstrong et al. 2004). Lim et al. (2006) reported that dietary supplementation of chitosan-Cu increased weight gain and feed intake in broilers. The present result was consistent with this study. Previous studies showed that high Cu had an "antibiotic-like effect" on the microflora in the intestinal tract of animals (Cromwell 2001). Mekahlia and Bouzid (2009) found that chitosan-Cu complex showed a wide spectrum of antibacterial activities against *Salmonella enteritidis in vitro*. This might be one of the reasons why dietary CS-Cu could decrease diarrhea incidence of pigs and had attractive biological function *in vivo*. As a kind of chelate compound of chitosan and Cu,

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CS-Cu may both have the functions of chitosan and Cu. However, feeding 100 mg Cu/kg diet as $CuSO_4$ or $CuSO_4$+CS did not improve growth rate as CS-Cu. This means the biological functions of CS-Cu do not simply come from the combination of CS and Cu.

Liver antioxidant enzyme activities. Though the antioxidant capacity of piglets fed dietary $CuSO_4$ or CS+$CuSO_4$ was higher than that of control pigs, it was much improved in pigs treated with CS-Cu. This means the biological function of CS-Cu is better than that of $CuSO_4$ or CS+$CuSO_4$ in pigs. The activity of these antioxidant enzymes is known to be decreased during Cu deficiency (Taylor et al. 1988). Therefore an increased availability of Cu may increase the activities of enzymes. In mammalian cells, H_2O_2 was usually converted from O_2^- by SOD and these enzymes are important as components in the antioxidant response system (Virgili et al. 1999). However, this study found that SOD and GPx activities were higher in pigs fed dietary CS-Cu than in those fed dietary $CuSO_4$. It is well-known that the mechanism prevents the formation of highly cytotoxic oxygen-derived free radicals. The increase of SOD activity may be due to the improvement of the stability or slowdown of degradation by itself (Wang et al. 2012). In addition, Cu, Fe, and Zn metal ions, the gross components of SOD in eukaryotic cells, could serve as co-factor to maintain the function of SOD. Perhaps copper in the form of organic could be absorbed more easily by the body than $CuSO_4$ and $CuSO_4$+CS. Furthermore, the increased level of copper could promote the absorption of zinc and iron and finally work together to improve the SOD activity.

Intestinal mucosal histology and ileal epithelial cells apoptosis. In the current study, Cu supplementation increased villus height, which was in agreement with Shurson et al. (1990) and in contrast to previous studies reporting no effect of high Cu supplementation on villus height (Radecki et al. 1992; Hedemann et al. 2006). It is well known that in piglets villus atrophy and crypt hyperplasia occur after weaning (Xiong et al. 2015). The earlier the weaning, the more prominent these morphological changes. Cu supplementation decreased crypt depth, which is in agreement with Hedemann et al. (2006) who evaluated the effect of dietary Cu and Zn during the 14-day post weaning period in pigs weaned at 28 days of age. However, this result was in contrast to previous studies showing increased crypt depths (Shurson et al. 1990) or no effect of higher dietary Cu intake on crypt depths (Radecki et al. 1992). Perhaps CS-Cu could increase the amount of readily available nutrients, and thus improve villus development and repair. Apoptosis occurred in pigs unsupplemented with Cu. This change might contribute to the injury of intestinal epithelium integrity, resulting in a compromise in its barrier function. However, apoptosis of epithelium cells decreased in pigs fed 100 mg CS-Cu/kg diet. These changes might be responsible for the observed effects on the small intestinal morphology.

In summary, current study showed that CS-Cu could improve growth performance and affect some physiological responses. It showed better biological activities than $CuSO_4$ or $CuSO_4$+CS. CS-Cu had a marked effect on diarrhea, small intestinal morphology, and the activities of antioxidant enzymes in liver. In spite of the marked changes observed in gut morphology when feeding CS-Cu, the current study does not give definite answers on how the growth promoting and diarrhea reducing effects of CS-Cu are exerted. CS-Cu may be a kind of Cu source for animal, and further studies are needed to elucidate the biological function of CS-Cu.

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