Asian-Aust. J. Anim. Sci.
Vol. 21, No. 10 : 1473 - 1478
October 2008

www.ajas.info

The Growth-promoting Effect of Tetrabasic Zinc Chloride is Associated with Elevated Concentration of Growth Hormone and Ghrelin

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ABSTRACT : An experiment was conducted to investigate the mechanism for the effect of tetrabasic zinc chloride (TBZC) in enhancing growth performance of weanling piglets. Gut-brain peptides play an important role in the regulation of growth and appetite in animals. This study evaluated the effects of TBZC on blood concentrations of growth hormone (GH), ghrelin, insulin-like growth factor-I (IGF-I), cholecystokinin (CCK) and neuropeptide Y (NPY). Seventy-two weanling piglets (Landrace×Large White) with an initial body weight (BW) of 6.7±0.16 kg and aged 24±1 days were assigned to three dietary treatments: i) control diet without TBZC supplement, ii) the control diet supplemented with 2,000 mg Zn from TBZC/kg and iii) TBZC-supplemented diet pair-fed with respect to the control diet. Each treatment had six replications (pens) of four piglets. At the end of a 14-d experimental period, piglets were weighed and feed consumption was measured, and blood samples were collected for assays of GH, ghrelin, IGF-I, CCK and NPY concentrations. The inclusion of TBZC in the diet increased average daily gain ($p<0.01$), average daily feed intake ($p<0.05$), and feed conversion ratio ($p<0.05$). Pair-fed piglets had higher ADG, and lower FCR than ($p<0.05$) Control piglets. Supplementation of the diet with TBZC increased ($p<0.05$) serum GH and plasma ghrelin levels in weanling piglets, but did not affect ($p>0.05$) serum IGF-I and plasma NPY and CCK concentrations. Pair-fed piglets had lower ($p<0.05$) serum GH levels than TBZC-supplemented piglets, but did not ($p>0.05$) differ from Control piglets. These data indicated that TBZC elevated the concentration of ghrelin and GH. This observation may partly explain the beneficial effects of TBZC on growth performance of weanling piglets. (**Key Words :** Tetrabasic Zinc Chloride, Pig, Growth Hormone, Ghrelin)

INTRODUCTION

Feeding high level of zinc (Zn) to weanling piglets improves growth performance (Hahn and Baker, 1993; Poulsen, 1995; Case and Carlson, 1999). Our previous experiment indicated that feeding pharmacological tetrabasic zinc chloride (TBZC) improved growth performance of weanling piglets (Zhang and Guo, 2007). However, the mechanism of this action remains unclear.

The growth hormone (GH)/insulin-like growth factor-I (IGF-I) axis plays a major role in the regulation of body growth in vertebrates (Rosenfeld and Roberts, 1999). The main hormones implicated in the GH/IGF axis are the pituitary GH and the liver-derived endocrine IGF-I. The IGF-I mediates the growth-promoting effects of GH, was produced by the hepatocyte in response to the binding of GH to the GH receptor (GH-R) (Yakar et al., 1999). Circulating ghrelin, the endogenous ligand of GH secretagogues-receptor (GHS-R), is synthesized primarily in the stomach in mammals (Bhatti et al., 2006). Ghrelin acts on the GHS-R, increasing intracellular Ca^{2+} levels via inositol 1,4,5-trisphosphate (IP3) to stimulate GH release (Kojima and Kangawa, 2005). The addition of dietary Zn to the weanling piglet diet enhanced serum IGF-I levels (Carlson et al., 2004). Researcher suggested that Zn enhanced the growth performance of weanling piglets through a direct influence on the gastrointestinal tract (Li et al., 2006).

Cholecystokinin (CCK) and neuropeptide Y (NPY) have been implicated in the control of feed intake (FI) in a number of species. Gastrointestinal (GI) peptide hormones, most notably CCK, are important factors that control appetite and satiety (Huda et al., 2006). The CCK released from the proximal small intestine, functions as a short-term satiation signal by inducing satiety and decreasing meal size (Havel, 2001). Specific areas in the hypothalamus and the brainstem are important in coordinating GI peptide

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Received January 23, 2008; Accepted April 16, 2008

Table 1. Composition of the basal diets

Ingredient	Amount (%)
Maize	55
Soybean meal (44% CP)	14
Extruded soybean meal	15
Fish meal	3
Sprayed dried plasma proteins	3
Soybean oil	1
Whey	4.8
Limestone	0.7
Dicalcium phosphate	1.1
Sodium chloride	0.35
Colistin	0.06
Choline chloride	0.2
L-lysine.HCl (78%)	0.28
DL-methionine	0.034
Threonine	0.04
Vitamin and mineral premix*	1.0
Wheat bran	0.436
Calculated composition	
DE (MJ/kg)	14.42
CP (%)	20.02
Ca (%)	0.75
Total phosphorus (%)	0.68
Available phosphorus (%)	0.51
Lysine (%)	1.33
Methionine and cystine (%)	0.74
Threonine (%)	0.89
Zn (mg/kg)	129.7

* Mineral and vitamin premix supplied (per kg of final diet): Zn, 100 mg ($ZnSO_4$); Cu, 10 mg ($CuSO_4 \cdot 5H_2O$); Fe, 100 mg ($FeSO_4$); Mn, 10 mg ($MnSO_4 \cdot H_2O$); Se, 0.3 mg (Na_2SeO_3); I, 0.5 mg (KI); vitamin A, 5,000 IU; vitamin D_3, 450 IU; vitamin E, 60 IU; vitamin K, 4.4 mg; riboflavin, 8.8 mg; niacin, 33 mg; pantothenic acid, 30 mg, vitamin B_{12}, 22 μg; folic acid, 900 μg; thiamin, 2.5 mg; pyridoxine, 4.0 mg; biotin, 200 μg.

hormones signals. The arcuate nucleus (ARC) in the hypothalamus contains neurones expressing NPY (Huda et al., 2006). In mammals, NPY is one of the most powerful orexigenic agents, and stimulates feed intake behavior (Stanley and Leibowitz, 1985). Feeding pharmacological level of TBZC increased FI of weanling piglets (Zhang and Guo, 2007).

Based on the information, we hypothesized that TBZC stimulated the secretion of gastric ghrelin, which signaled its effects to the hypothalamus and stimulated GH release, and eventually, IGF-I mediated the growth-promoting effect. Simultaneously, we investigated the circulating CCK and NPY levels to address whether the increase of FI by addition of TBZC was related to the alteration in CCK or NPY.

MATERIALS AND METHODS

Pigs and diets

Seventy-two weanling piglets (Landrace×Large White) with an initial body weight (BW) of 6.7±0.16 kg and 24±1 days of age were allotted to pens on the basis of similar BW, ancestry, and gender. Each treatment had six replications (pens) of four piglets (half castrated males and half females). Piglets were housed on plastic slotted floors (1.3 m×1.2 m per pens) with self-feeders, and automatic stainless nipple waterers. Feed and water were available for *ad libitum*. Temperatures (25-28°C) and a cycle of 16 h light: 8 h dark were controlled. The basal diet (Table 1) with approximately 129.7 mg Zn/kg was formulated to meet or exceed the nutrient requirements recommended by NRC (1998). The three experimental diets were: 1) control diet (Control) without TBZC supplemented, 2) the control diet supplemented with 2,000 mg Zn from TBZC/kg (TZn) and 3) diet TZ-supplemented pair-fed with respect to the control diet (Pair-fed). To separate effects of FI from effects of dietary TBZC, we used the TZ-supplemented piglets which were pair-fed to the control group of piglets, as described by Swamy et al. (2004). Briefly, the amount of feed consumed by the piglets fed the control diet was recorded daily. The pair-fed piglets received the same amount of feed that was consumed by the control group the previous day. Pair-fed piglets received the diet twice daily. The TBZC [$Zn_5(OH)_8Cl_2 \cdot H_2O$] used containing 58% Zn was provided by Xingjia Bio-engineering Co. Ltd., China and replaced wheat bran in the diet. The experiment lasted 14 days.

Sample collection

Faecal scores were evaluated daily and expressed as percentage for a period of 2 weeks. At the end of the experiment, piglets were weighed after overnight fasting and feed consumption was measured and the average daily gain (ADG), average daily FI (ADFI) and the feed conversion ratio (FCR) were measured. After overnight fasting, one barrow from each pen was selected randomly to collect blood samples via the anterior vena cava. One of blood sample was collected in containing aprotinin and EDTA-coated vacutainer tubes, immediately cooled in ice. Plasma was obtained by centrifugation at 2,500×g for 10 min at 4°C. A second portion blood was collected in containing aprotinin vacutainer tubes, allowed to coagulate for 30 minutes at 4°C. Subsequently, serum was separated by centrifuged at 2,500×g for 10 min at 4°C. The sample was quickly frozen in liquid nitrogen and then determined GH, ghrelin, IGF-I, NPY and CCK concentration.

Hormone assays

After extraction in reverse phase C18 columns, plasma was measured with a commercial ghrelin radioimmunoassay (RIA) kit (Phoenix Pharmaceutical, Inc., Belmont, CA, USA). The RIA kit uses a polyclonal antibody that recognizes octanoylated and non-octanoylated ghrelin and ^{125}I-ghrelin as a tracer molecule. Thus, this RIA kit detects total ghrelin. The inter- and intra-assay

Table 2. Growth performance and faecal scores of weanling piglets fed diets with or without supplemental TBZC*

Items	Control	TZn	Pair-fed	SEM	p-value
ADG (g)	219[c]	265[a]	241[b]	5.8	0.001
ADFI (g)	357[b]	383[a]	357[b]	4.9	0.039
FCR	1.63[a]	1.45[b]	1.49[b]	0.033	0.038
Faecal scores (%)**	17.76[a]	3.08[b]	3.17[b]	0.380	<0.001

* Each value represents the mean of six pens of four piglets.

** Faecal scores (%) were calculated as the percent of the total number of days that signs of scours were evident within the pen on the total number of days (56 d).

[a, b, c] Means on the same row lacking a common superscript letters are different ($p<0.05$).

TBZC = Tetrabasic zinc chloride; ADG = Average daily growth; ADFI = Average daily feed intake; FCR = Feed conversion ratio; Control = Control diet; TZn = Supplemented with 2,000 mg Zn from TBZC/kg; Pair-fed = Pair-fed with respect to the control diet.

coefficients of variation were 7.6% and 5.0% respectively. Assay sensitivity was 0.7 pg/ml. Serum IGF-I concentrations were measured by a commercially available human RIA kit (Diagnostic System Laboratories, Texas, USA). For separating IGF-I from its binding protein, an acid-ethanol precipitation technique was used (Daughaday et al., 1980). Samples were added directly to tubes containing high-affinity free IGF-I antibody and ^{125}I-labeled antibody, incubated for 20 h at 4°C, washed, directed to a second epitope, washed, and counted. Assay standards are recombinant human IGF-I: 10 to 1,000 ng/ml. The sensitivity of the assay was 1.3 ng/ml. The intra- and inter-assay coefficients of variation were 3.5% and 7.9%, respectively. Serum GH was measured by RIA (Amersham Pharmacia Biotech, Little Chalfont, UK). The sensitivity of plasma GH was 0.35 ng/ml. The intra- and inter-assay coefficients of variation were 6.8% and 3.9%, respectively. For the determination of NPY levels in plasma, samples were extracted with HCl-ethanol (15:1,000, v/v) in a 1:2 ratio (plasma:HCl-ethanol) according to the method of Bauer-Dantoin et al (1991). Plasma NPY concentrations were measured by RIA kits (Peninsula, Belmont, CA, USA) after plasma extraction. The intra- and inter-assay coefficients of variation were 2.9% and 11%, respectively. Plasma CCK was extracted by a Sep-pak C18 cartridge (Waters, Milford, MA), and frozen at -80°C for determination of CCK concentrations. Plasma CCK concentrations were measured by RIA assay (Department of Endocrinology Royal Postgraduate Medical School, London, UK).

Statistical analysis

Data were subjected to Levene's homogeneity of variances test before the analysis. All p-value for homogeneity of variances test were higher than 0.05. Data were analyzed by ANOVA using the General Linear Model (GLM) procedures of SAS (1999. SAS Inst., Inc., Cary, NC). Pen was considered the experimental unit. The significance of mean differences between treatments was detected by the Duncan's multiple-range test. Differences were considered significant at $p<0.05$. Scouring data were analyzed after being arcsin transformed. Actual scouring data listed in the table, but SEM was for the transformed data.

RESULTS

Growth performance and faecal scores

Inclusion of TBZC at 2,000 mg Zn/kg diet increased ADG ($p<0.01$), ADFI, and FCR ($p<0.05$) of weanling piglets compared with Control (Table 2). In a previous experiment, Zhang and Guo (2007) indicated that dietary supplementation with TBZC increased ADFI in weanling piglets, and therefore, we conducted a pair-fed treatment to shun the influence of TBZC supplementation on FI. As shown in Table 2, Pair-fed piglets had higher ADG, and lower FCR than ($p<0.05$) Control piglets. However, ADG was decreased ($p<0.05$) when compared with TZn piglets. Supplementation of Zn from TBZC reduced the faecal scores. TZn and Pair-fed piglets had lower ($p<0.001$) faecal scores than Control piglets.

GH, IGF-I, and ghrelin concentrations

The effect of treatment on GH, ghrelin, and IGF-I was shown in Table 3. Supplementing TBZC to the diet (TZn) increased ($p<0.05$) serum GH and plasma ghrelin levels in weanling piglets, but did not affect ($p>0.05$) serum IGF-I concentration. Pair-fed piglets had lower ($p<0.05$) serum GH levels than TZn piglets, but did not ($p>0.05$) differ with Control piglets. Pair-fed piglets had a same plasma ghrelin levels as TZn piglets, but higher ($p<0.05$) than Control piglets.

NPY and CCK concentration

Supplementation of TBZC into the diet didn't affect ($p>0.05$) plasma NPY and CCK concentration (Table 3).

DISCUSSION

Adding TBZC to the diet enhanced growth performance of weanling piglets for week 1 and 2. This confirms previous reports that supplementation of Zn at 1,500 to 3,000 mg/kg diet from TBZC increased ADG of weanling piglets (Mavromichalis et al., 2001; Zhang and Guo, 2007).

Table 3. GH, ghrelin, IGF-I, CCK and NPY levels in blood of weanling piglets fed diets with or without supplemental TBZC*

Items	Control	TZn	Pair-fed	SEM	p-value
GH (ng/ml)	3.05 [b]	4.14 [a]	3.60 [b]	0.198	0.072
Ghrelin (pg/ml)	547.7 [b]	621.7 [a]	616.1 [a]	13.30	0.029
IGF-I (ng/ml)	171. 9	169.3	176.3	7.94	0.944
CCK (pmol/L)	2.41	2.07	2.77	0.299	0.872
NPY (pg/ml)	140.0	132.0	139.8	6.32	0.855

* Each value represents the mean of six pens of one piglet.

[a, b] Means on the same row lacking a common superscript letters are different ($p<0.05$).

GH = Growth hormone; IGF-I = Insulin-like growth factor-I; CCK = Cholecystokinin; NPY = Neuropeptide Y; TBZC = Tetrabasic zinc chloride.

Control = Control diet; TZn = Supplemented with 2,000 mg Zn from TBZC/kg; Pair-fed = Pair-fed with respect to the control diet.

The pair-fed group indicated that the enhanced growth response was not solely due to increase voluntary FI, but also to improve feed efficiency. The current experiment suggested that supplementation of TBZC at the level of 2,000 mg/kg to piglet diets resulted in lower faecal scores. This result is consistent with our previous reports that high levels of TBZC reduced the incidence and severity of diarrhea, and improved faecal consistency after weanling (Zhang and Guo, 2007).

However, the underlying mechanism, by which the high level of Zn enhanced growth performance of weanling piglets, is still a matter of controversy. Hahn and Baker (1993) indicated that high levels of ZnO enhanced growth performance of weanling piglets through a systemic effect, and that the Zn ion is a major causative factor. Surprisingly, Mavromichalis et al. (2001) found that high (93%) or low (39%) bioavailability of ZnO did not substantially influence the growth-promoting efficacy. In addition, the injection of Zn around weaning did not have a positive effect on growth performance of weanling piglets, although serum Zn concentrations tended to increase (Schell and Kornegay, 1994). Thus, this speculation about the mechanism responsible for the growth-promoting effects of high level Zn on weanling piglets was not reasonable. The GI tract (GIT) plays an important role where nutrients and physiological signals are exchanged between the inside and outside of the body, which influences animal digestion, absorption and metabolism, and thereby determines body growth, development and health condition. The TBZC enters into the GIT, and may affect the secretion of GI hormones. This leads to the hypothesis that the addition of TBZC to weanling piglets diets results in higher gastric ghrelin, which in turn results in increasing GH, and eventually improving growth performance.

Ghrelin is synthesized and released primarily by endocrine X/A cells in the stomach (Kojima et al., 1999; Bhatti et al., 2006) and plays an important role in the control of GH secretion. Kojima et al. (1999) found that ghrelin specifically stimulated GH release, but did not affect other pituitary hormones. Consistent with this notion, we found that the concentration of circulating GH and ghrelin was increased by the addition of TBZC to weanling piglet diet. The serum GH levels of Pair-fed piglets did not differ with Control piglets, but had a trend elevation. Therefore, this may demonstrate that the inducement of the pituitary GH secretion was due to the effect of TBZC on gastric ghrelin. However, there are many factors that affected blood ghrelin concentration, and only the total ghrelin was measured in the current experiment. Thus, the hypothesis that TBZC stimulated the secretion of gastric ghrelin, which signaled its effects to the hypothalamus and stimulated GH release, was not well established. Further studies are required to determine active ghrelin (acylated ghrelin) and to elucidate the molecular mechanism whereby of TBZC regulates the secretion of gastric ghrelin.

The current experiment suggested that serum IGF-I concentrations were not affect by the addition of TBZC to diet, which agreed with Li et al. (2006) reporting that inclusion of 3,000 mg Zn/kg from ZnO in the diet did not affect serum IGF-I concentrations in weanling piglets. In contrast, Carlson et al. (2004) reported that additional dietary ZnO in weanling diets for piglets increased serum IGF-I. This discrepancy may be due to the different source of Zn, the age of piglets and methodology of blood sampling. Sjōgren et al. (1999) indicated that liver-derived IGF-I was not required for postnatal body growth. Several studies have shown that GH has independent activities (Clark et al., 1994; Guler et al., 1988). The current experiment may suggest that the growth-promoting effects of TBZC was the result of a direct action of GH, and that it was not mediated by IGF-I. Previous reports indicated that liver-derived IGF-I exerts a negative feedback-regulation of GH secretion by suppression of GH-releasing hormone receptor expression in the hypothalamic (Wallenius et al., 2001). The lack of effect of TBZC supplementation on plasma IGF-I may be beneficial for the independent activities of GH.

Peripheral signals regulating appetite originate primarily from the GI tract. Ghrelin and CCK are the two most important peripheral signals. These peripheral signals modulate the release of appetite-related neuropeptides in the brain, and consequently feed intake. The effect of TBZC on circulating CCK and NPY concentrations has been tested for first time. There was no effect on circulating CCK and NPY levels in current experiment, suggesting that the increase of feed intake by TBZC was not related to the

CCK and NPY pathways.

Ghrelin contributes to the acute regulation of appetite and satiety. It had been shown to induce hunger and increase FI (Huda et al., 2006). Ghrelin exerts its feeding activity by stimulating NPY/AgRP neurons in the hypothalamus to promote the production and secretion of NPY and AgRP peptides (Kojima and Kangawa, 2005). In the current experiment, only the blood NPY level was determined; whereas the changes in concentration of NPY in specific brain areas responding to TBZC were not studied. However, Tschöp et al. (2004) and Wang et al. (2008) indicated that ghrelin seems to stimulate energy metabolism and increase BW more rapidly and more potently than it affects feed intake. Wu et al. (2008) indicated that ghrelin infusion did not significant influence on feed intake. Further studies are required to verify whether the increase of FI by TBZC was mediated by ghrelin, and elucidate its mechanism.

In conclusion, dietary supplementation with a high level of TBZC increased blood ghrelin and GH. This novel finding may partly explain the beneficial effects of TBZC on growth performance of weanling piglets.

ACKNOWLEDGMENT

This work was financially supported by the National Basic Research Program, Project No. 2004 CB 117504. We thank Bun Sidoeun, Xi He, Yi Wang, Xiaojun Yang, Xiaofei Hu, Jinlei Wen, Xin Yang, Haiying Wang, Zhonghong Hu and Jian Zhao for excellent technical assistance in the experiments.

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