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ORIGINAL PAPER

Effects of active immunization against cholecystokinin 8 on performance, contents of serum hormones, and expressions of CCK gene and CCK receptor gene in pigs

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Abstract This study was conducted to investigate the effects of active immunization against cholecystokinin 8 (CCK_8) on the content of serum CCK, expression of CCK, and CCK receptor gene in pigs. The subjects for this experiment were 15 pigs divided into three groups (5 pigs per group). The treated groups were immunized with CCK_8 conjugated to human serum albumin (HSA). The control group was immunized with same dosage of HSA. The average daily gain of pig fed with 250 μg CCK was significantly increased ($P < 0.05$), compared with the control group (0 μg CCK). The content of CCK_8, insulin, and leptin in serum was significantly ($P < 0.05$) decreased and the titer of CCK_8 antibody was significantly ($P < 0.05$) increased in treated groups compared to the control group. The levels of CCK gene and CCK receptor gene expression in jejunum, pituitary, and pancreas of the treated groups were significantly ($P < 0.05$) lower than that of the control group. It is concluded that optimal active immunization against CCK_8 could increase the content of CCK antibody and suppress CCK gene and CCK receptor gene expressions and in result improve feed intake and growth performance of pigs.

Keywords Active immunization · CCK_8 · CCK gene · CCK receptor gene · Pig

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Introduction

Cholecystokinin (CCK), a well-known satiety signal molecular, is an important brain-gut peptide [1, 2]. CCK is distributed both throughout the brain [3] and the gastrointestinal tract [4]. CCK appears to be involved in the modulation of pancreatic enzyme release and the inhibition of feeding and gastric emptying [1, 2, 5].

It is well established that small peptides that conjugate to high-molecular weight carrier proteins can be rendered more immunogenic, and that immunogenicity of such carrier proteins differs between species of animals [6]. A number of molecular forms of CCK have been identified [7, 8]. CCK_8 is suggested to be the shortest form displaying the full biological activity [9]. Active immunization against porcine CCK_8 was shown to increase feed intake and growth of pigs [10, 11], which could be further improved by increasing the immune response and titer of CCK_8 antibody. The antibodies produced are thought to neutralize endogenous cholecystokinin [10, 11].

In vivo, CCK release is stimulated by luminal nutrients, such as fat [12] and protein [13]. In vitro study demonstrated that the transcription level of CCK gene was increased by pituitary adenylate cyclase-activating polypeptide (PACAP) in STC-1 cells [14], suggesting that external factors can affect the secretion of CCK and the expression of CCK gene. CCK is shown to elicit its satiating function by binding to CCK receptor [1, 15]. However, the effects of active immunization against CCK_8 on the CCK gene and CCK receptor gene expression remain largely unknown.

In this study, we investigated the effects of active immunization against CCK_8 on the content of serum CCK, expressions of CCK gene, and CCK receptor gene in pigs.

Our data showed that CCK-induced suppression of feed intake was decreased by active immunization against CCK. This work reveals for the first time the effects of active immunization against CCK_8 on feed intake in pigs from immune and molecular levels.

Materials and methods

Animals and treatment

The experiment was designed as a single factor. Fifteen Duroc × Danish Landrace × Large White (DLY) cross-bred pigs with average weight of 27.66 ± 1.71 kg were randomly divided into three treatments. Pigs in treatment 1, 2, and 3 were immunized with 650 μg HAS + 0 μg CCK_8, 650 μg HAS + 250 μg CCK_8, and 650 μg HAS + 500 μg CCK_8, respectively. Each treatment had five replicates with one pig per replicate. The pigs were fed corn-soybean meal—extruded soya diet formulated according to NRC (1998) requirements (Table 1). The test period lasted for 75 days.

Table 1 Composition and nutrient levels of the basal diet (% as fed)

	Stage	
	20–50 kg	50–100 kg
Ingredient (%)		
Corn	72.7	79.28
Extruded soya	9	7
Soybean meal	16.1	12
Salt	0.3	0.3
Choline chloride	0.1	0.1
Dicalcium phosphate	0.6	0.54
Limestone	0.63	0.3
L-Lys	0.23	0.15
Vitamin premix[a]	0.04	0.03
Trace mineral premix[b]	0.3	0.3
Nutrition level[c]		
ME (Mcal/kg)	3.33	3.27
CP (%)	16.05	14.07
Dig-Lys (%)	0.84	0.66
Ca (%)	0.62	0.49
NP (%)	0.23	0.19

[a] Composition (per kilogram of diet): vitamin A, 19,000 IU; vitamin D_3, 3,860 IU; vitamin E, 36.7 IU; vitamin K_3, 3.6 mg; vitamin B_1, 15 mg, vitamin B_2, 15 mg; vitamin B_6, 5 mg; vitamin B_{12}, 0.05 mg; biotin, 0.1 mg; folic acid, 2.5 mg; niacin, 73.3 mg; calcium panthenate, 25 mg

[b] Composition (g/kg of diet): Fe, 160; Cu, 10; Zn, 100; Mn, 20; Se, 0.3; I, 0.15

[c] Nutrition level value was calculated

Preparation of antigen and immunization of pigs

The antigen was prepared according to the method described by Pekas and Trout [10]. Immunization with cross-linked polymer of CCK_8 and HSA was carried out with an initial injection of 250 μg CCK_8 (or 500 μg CCK_8) (Sigma, C2901) and 650 μg HSA. The immunization schedule was continued by three multisite intramuscular injections at days 29, 43, and 59 of the experiment, respectively. The control group was treated with injection of same amount of HSA.

Sample collection

Blood samples were collected via the superior vena cava on days 1, 15, 29, 43, 59, and 73. The blood tubes were immediately centrifuged at 5000 × *g* at 4°C for 5 min and stored at −20°C before analyzing the antibody titers. The serum from days 1, 43, and 73 were used to determine the content of CCK_8, insulin, and leptin. At the end of the trail, the pituitary, a 5–8 cm of the proximal jejunum, and segmental pancreas were immediately removed, frozen in liquid nitrogen, and stored at −70°C for determination of the mRNA content of CCK gene and CCK receptor gene.

Feed intake and growth rate

Pigs were individually weighed at the end of experiment. Feed intake was recorded once a week. Average daily gain (ADG, g/day) and average daily feed intake (ADFI, g/day) for the whole period were calculated and statistically analyzed.

Antibody titers of CCK_8 in serum

Serum antibody titer of CCK_8 was detected by indirect ELISA. The polystyrene microplate was irradiated by ultraviolet, activated by glutaraldehyde prior to coating, and added with 150 μl of CCK_8 to each well of the microplates at a concentration of 20 μg/ml in coating buffer. Then, the plate was sealed with plate sealer and incubated at 4°C for overnight. After washing three times with wash buffer, the plate was incubated at 37°C for 2 h with the diluted serum, negative serum, and positive serum (1:10). After washing, the plate was incubated at 37°C for 3 h with Staphylococcal protein A (SPA, Institute of Kexin Biotechnology, Shanghai, China). The reaction was visualized by using O-phenylenediamine (OPD) substrate. Fifteen minutes later, the substrate reaction was terminated by adding 37 μl/well of stop solution. Subsequently, the plate was cooled to room temperature and an enzyme mark apparatus (Thermo, USA) was used to measure the

absorbance at 490 nm. Each standard and unknown sample was measured in triplicate.

The content of hormone in serum

The CCK_8 concentration in serum was measured using a CCK Octapeptide (Human) (Non-Sulfated) ELISA immunoassay kit (Phoenix, USA). Insulin level was determined using the DSL-10-1600 ACTIVE^{TM} Insulin ELISA Kit (Diagnostic Systems Laboratories, Inc., Webster, TX, USA). Serum leptin level was measured using ACTIVE Human Leptin ELISA Kit (DSL-10-23100, Diagnostic Systems Laboratories, Inc., Webster, TX, USA). The methods and operations were performed according to the manufacturer's instructions.

Northern hybridization analysis

Primers for CCK gene and CCK receptor gene were designed according to the porcine CCK gene (5′-GGGGCACAG GAGG AGGAGGCG-3′) (GenBank accession no. K01940) and human CCK receptor gene (5′-GGGCGAGGGGA GAGGTGGGGG-3′) (GenBank accession no. NM000730). All primers were synthesized by Shanghai Sangon Biotechnology Corporation (China). Total RNA from the jejunum, pituitary, and pancreas were extracted according to the manufacturer's instructions. RNA samples were separated by electrophoresis in a 1% agarose gel containing formaldehyde and then transferred to a Hybond-N^+ nylon membrane (Amersham). Hybridization was performed using photobiotin-labeled cDNA probe. The optical density of hybrid brand was detected using a GeneTool image analyzer software. Each experiment was repeated 4 times.

Statistical analysis

Data were analyzed by ANOVA using the General Linear Model (GLM) procedure of SPSS 10.0 (SPSS Inc., Chicago, IL), and the LSD was used to put up multiple comparisons. Results were expressed as mean ± SD. The level of statistical significance was set at $P < 0.05$.

Results

Effects of active immunization against CCK on growth performance

The performance of pigs from each treatment is presented in Fig. 1. There was a significant difference in average daily gain during the whole period in pigs administrated with 250 μg CCK, compared with the pigs with 500 μg CCK and control group ($P < 0.05$). There was no statistically significant difference between pigs with 500 μg CCK and control group. A similar result was observed in average daily feed intake during the whole period (Fig. 1).

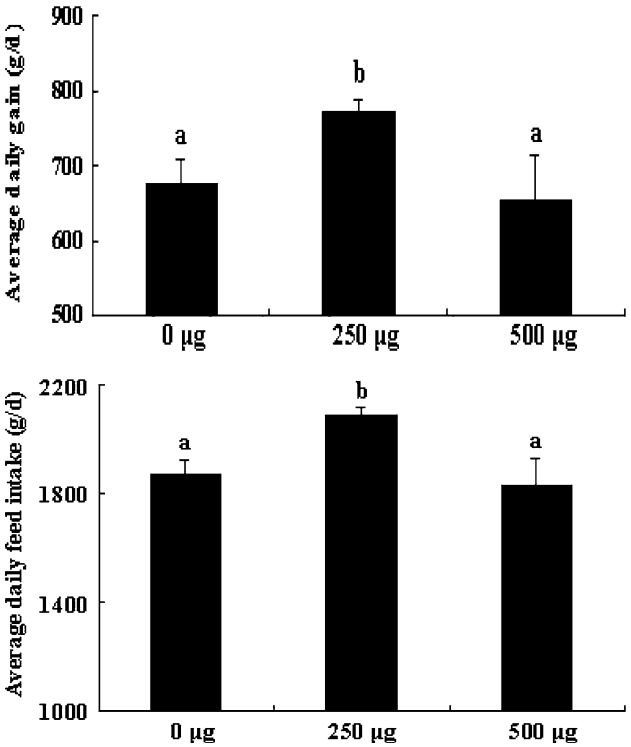

Fig. 1 The effects of active immunization against CCK on the growth performance of pigs. Results were the mean and standard deviation. Bar with different letter indicated significant difference ($P < 0.05$)

Effects of active immunization against CCK_8 on the titer of CCK_8 antibody in serum

Indirect ELISA analysis revealed that the titers of CCK_8 antibody in serum from pigs administrated with 250 μg CCK ($P < 0.05$) and 500 μg CCK ($P < 0.05$) were larger than that of the control group (Fig. 2) on day 43. There was an extreme significant difference between treated groups and control group on days 59 and 73 ($P < 0.01$). No significant difference was observed in the titer of CCK_8 antibody between two treated groups (Fig. 2).

Effects of active immunization against CCK_8 on the content of CCK_8 in serum

On day 43, the contents of serum CCK_8 in pigs with 250 and 500 μg CCK were decreased by 16.26% ($P = 0.07$) and 21.05% ($P < 0.05$), respectively, compared to the

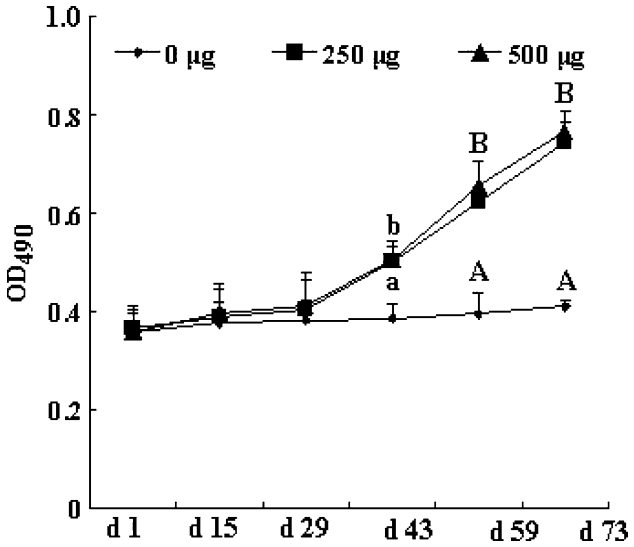

Fig. 2 The effects of active immunization against CCK_8 on the titer of CCK_8 antibody in serum. Results were the mean and standard deviation. Bar with small alphabet indicates significance of difference under 5% level ($P < 0.05$). Bar with big alphabet indicates significance of difference under 1% level ($P < 0.01$)

control group (Fig. 3). On day 73, the contents of serum CCK_8 in treated groups were decreased by 29.0% ($P < 0.05$) and 37.72% ($P < 0.05$) compared with the control group (Fig. 3). There was no significant difference between treated groups and control group on day 1 (Fig. 3).

Effects of active immunization against CCK_8 on hormone in serum

On day 43, the contents of serum insulin in pigs with 250 and 500 μg CCK were decreased by 12.36% and 12.71%, respectively, compared to the control group (Fig. 4). On

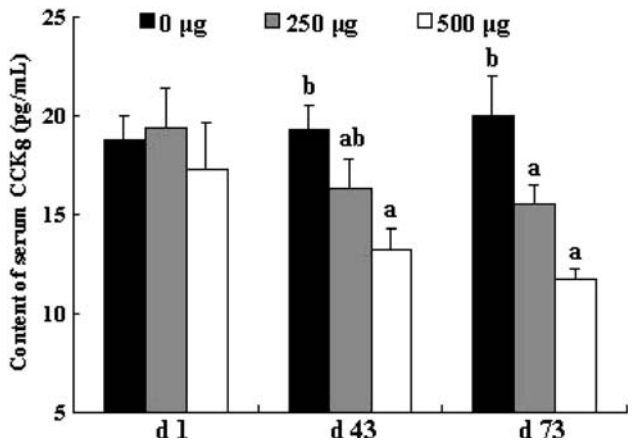

Fig. 3 The effects of active immunization against CCK_8 on the content of CCK in serum. Results were the mean and standard deviation. Bar with different letter indicated significant difference ($P < 0.05$)

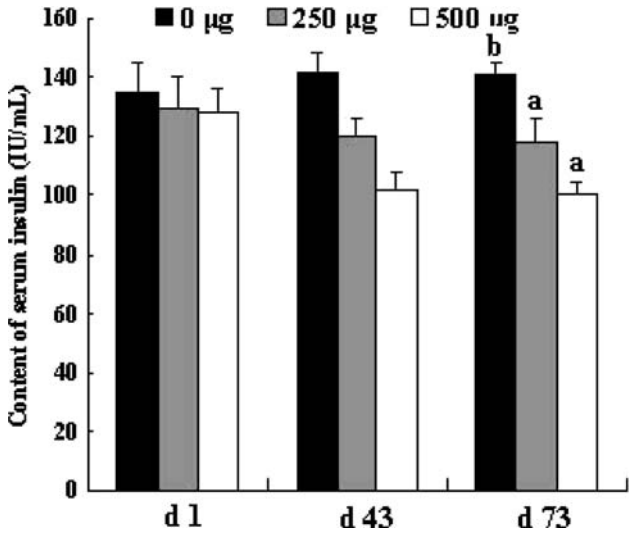

Fig. 4 The effects of active immunization against CCK_8 on the content of insulin in serum. Results were the mean and standard deviation. Bar with different letter indicated significant difference ($P < 0.05$)

day 73, the contents of serum insulin in treated groups were decreased by 21.39% ($P < 0.05$) and 23.57% ($P < 0.05$) compared with the control group (Fig. 4). There was no significant difference between treated groups and control group on day 1 (Fig. 4). These results suggested that active immunization against CCK_8 could decrease the content of insulin in serum.

On day 43, the contents of serum leptin in pigs with 250 and 500 μg CCK were decreased by 23.59% ($P < 0.05$) and 22.26% ($P < 0.05$), respectively, compared to the control group (Fig. 5). A similar result was observed on

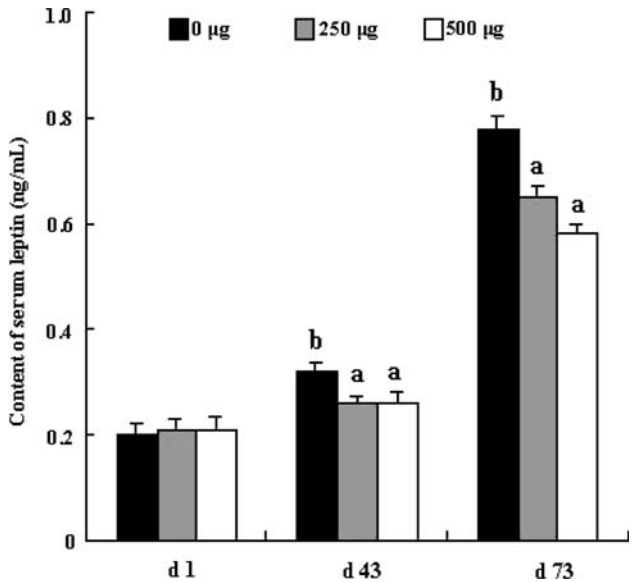

Fig. 5 The effects of active immunization against CCK_8 on the content of leptin in serum. Results were the mean and standard deviation. Bar with different letter indicated significant difference ($P < 0.05$)

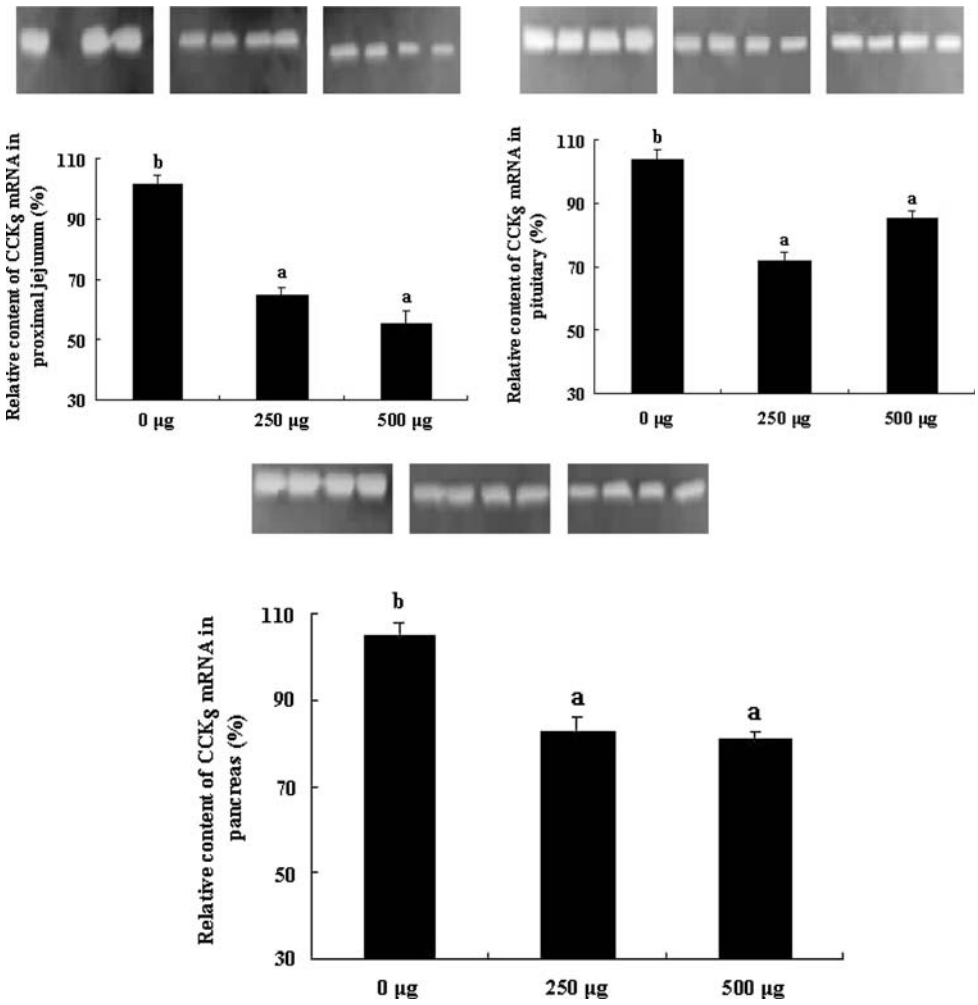

Fig. 6 The effects of active immunization against CCK_8 on the CCK gene expression. Results were the mean and standard deviation. Bar with different letter indicated significant difference ($P < 0.05$)

day 73 (Fig. 5). There was no significant difference between treated groups and control group on day 1 (Fig. 5).

Effects of active immunization against CCK_8 on the CCK gene and CCK receptor gene expression

Northern hybridization analysis demonstrated that the CCK mRNA levels in treated groups ($P < 0.05$) were lower than that of the control group in pituitary, jejunum, and pancreas. No significant difference was observed between treated groups (Fig. 6). A similar result was observed in CCK receptor gene expression (Fig. 7). These results revealed that the active immunization against CCK_8 could affect the endogenous CCK gene and CCK receptor gene expression.

Discussion

It has been reported that CCK could be able to suppress feed intake and induce satiety of animals [16–20]. Active immunization against CCK was shown to improve feed intake and growth performance of pigs [10, 11, 21, 22]. In this study, we showed that pigs immunized with 250 μg of CCK_8 indeed increase the feed intake and average daily gain of pigs. We also provided evidence here that pigs immunized with CCK_8 could decrease the content of serum CCK and increase the content of CCK antibody in serum. These results suggested that the effect of active immunization against CCK on the feed intake in pigs may be due to the increase in the content of CCK_8 antibody, which neutralize endogenous CCK of pigs.

However, the feed intake and average daily gain in pigs fed with 500 μg of CCK_8 were reduced ($P > 0.05$) compared with control group in this study. It may be due to the immunological stress and pathologic changes of pancreas tissue [23, 24]. Indeed, we observed that active immunization against 500 μg of CCK_8 resulted in focal vacuolar degeneration in porcine pancreas gland cells, the structural damage of pancreatic acinar cells, and the congestion and hemorrhage in the necrotic and metamorphic region (unpublished data). It is well known that CCK plays

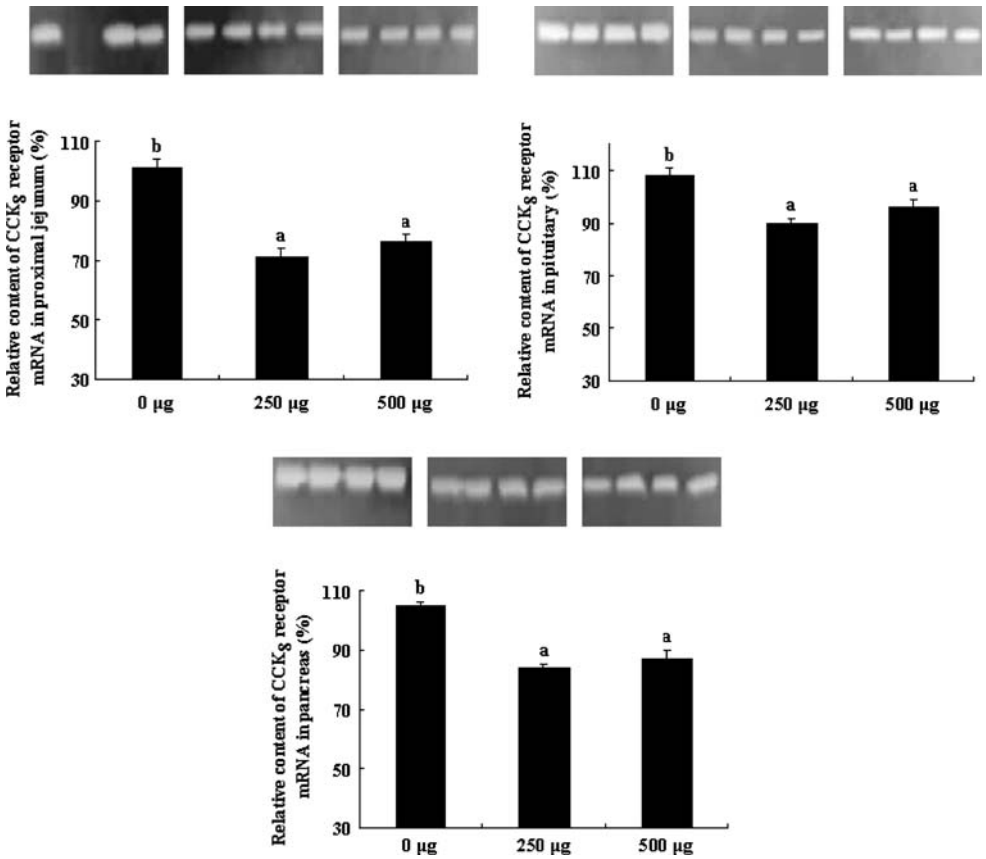

Fig. 7 The effects of active immunization against CCK_8 on the CCK receptor gene expression. Results were the mean and standard deviation. Bar with different letter indicated significant difference ($P < 0.05$)

important role in pancreas development and functions. The present study indicated that active immunization against CCK has two sides of biological effects. One is to weaken the inhibiting effect of CCK on feed intake; the other is to have negative impacts on pancreas function. Therefore, the extent of active immunization is essential for regulating feed intake without negative effect on other functions of pancreas. This study showed that immunization with 250 μg of CCK_8 instead of 500 μg of CCK_8 is optimal.

The satiation signals from the gut are regulated by hormones, such as insulin and leptin. Insulin is one of the factors in regulating animal feed behavior. CCK has been reported to enhance insulin release [5]. In our study, we found the content of insulin in serum was significantly decreased by active immunization against CCK_8. Leptin is suggested to synergistically interact with the CCK to control satiety in animals [25, 26]. Here we found that active immunization against CCK_8 significantly decreased the content of leptin in serum. These results suggested that the regulation of feed intake in pigs by active immunization against CCK_8 may be due to the synergistical interactions of CCK, insulin, and leptin.

CCK functions by binding to CCK receptor [1, 15]. Two types of CCK receptors (CCK-A and CCK-B) have been identified. CCK-A is suggested to play a dominant role in CCK-mediated suppression of feed intake [27]. Stimulation of CCK release from peripheral receptor of gastrointestinal wall after a meal is followed by an increase in CCK-A receptor at vagal afferent nerve fibers terminals and enhancement of CCK-induced satiety in the magnocellular neuroendocrine neurons in paraventricular nucleus of the hypothalamus (PVN) and suppression of feed intake [28–30]. Our data revealed that CCK gene and CCK receptor gene expression of pigs in pituitary, jejunum, and pancreas was significantly decreased by active immunization against CCK_8. These results suggested that the signal satiety passway of CCK via sensory fibers of the vagus nerve to the brain was decreased, which lowers CCK-induced suppression of feed intake, increase the feed intake in pigs, and improve the growth performance of pigs.

In summary, our findings demonstrated that active immunization against CCK_8 could increase the feed intake from immune and molecular levels. Active immunization against CCK_8 was also demonstrated to increase the content of CCK antibody and suppress CCK gene and CCK receptor gene expression in proximal jejunum, pituitary, and pancreas, which results in the decrease of CCK_8 content in serum, the attenuation of CCK-induced suppression of feed intake in pigs, and increment in growth performance of pigs.

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References

1. T.H. Moran, K.P. Kinzig, Gastrointestinal satiety signals II. Cholecystokinin. Am. J. Physiol. Gastrointest. Liver Physiol. **286**, G183–188 (2004)
2. A.D. Strader, S.C. Woods, Gastrointestinal hormones and food intake. Gastroenterology **128**, 175–191 (2005)
3. M.C. Beinfeld, M. Palkovits, Distribution of cholecystokinin in the hypothalamic limbic system of the rat. Neuropeptides **2**, 123–128 (1981)
4. L.I. Larsson, J.F. Rehfeld, Distribution of gastrin and CCK cells in the rat gastrointestinal tract. Evidence for the occurrence of three distinct cell types storing COOH-terminal gastrin immunoreactivity. Histochemistry **58**, 23–31 (1978)
5. J.H. Walsh, Gastrointestinal hormones, in *Physiology of the Gastrointestinal Tract*, ed. by L.R. Jouhnson (Raven, New York, 1994), pp. 1–128
6. E. Harlow, D. Lane, *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988)
7. J. Eng, Y. Shiina, Y.C. Pan, R. Blacher, M. Chang, S. Stein, et al., Pig brain contains cholecystokinin octapeptide and several cholecystokinin desoctapeptides. Proc. Natl. Acad. Sci. **80**, 6381–6385 (1983)
8. U. Gubler, A.O. Chua, D. Young, Z.W. Fan, J. Eng, Cholecystokinin mRNA in porcine cerebellum. J. Biol. Chem. **262**, 15242–15245 (1987)
9. C. Yanaihara, A. Kuwahara, M. Suzuki, M. Hoshino, M. Li, L.Q. Zheng, et al., Novel CCK analogs for studying CCK-B receptor. Ann. N. Y. Acad. Sci. **713**, 107–117 (1994)
10. J.C. Pekas, W.E. Trout, Stimulation of food intake and growth of swine by cholecystokinin immunization. Growth Dev. Aging **54**, 51–56 (1990)
11. J.C. Pekas, W.E. Trout, Cholecystokinin oktapeptide immunization: effect on growth of barrows and gilts. J. Anim. Sci. **71**, 2499–2505 (1993)
12. G.M. Green, S. Taguchi, J. Friestman, W.Y. Chey, R.A. Liddle, Plasma secretin, CCK, and pancreatic secretion in response to dietary fat in the rat. Am. J. Physiol. **256**, G1016–1021 (1989)
13. R.A. Liddle, G.M. Green, C.K. Conrad, J.A. Williams, Proteins but not amino acids, carbohydrates, or fats stimulate cholecystokinin secretion in the rat. Am. J. Physiol. Gastrointest. Liver Physiol. **251**, G243–248 (1986)
14. D.G. Deavall, R. Raychowdhury, G.J. Dockray, R. Dimaline, Control of CCK gene transcription by PACAP in STC-1 cells. Am. J. Physiol. Gastrointest. Liver Physiol. **279**, G605–612 (2000)
15. T.H. Moran, E.E. Ladenheim, Identification of receptor populations mediating the satiety actions of brain/gut peptides, in *Satiation: From Gut to Brain*, ed. by G.P. Smith (Oxford University Press, NY, 1998), pp. 126–163
16. J. Gibbs, R.C. Young, G.P. Smith, Cholecystokinin decreases food intake in rat. J. Comp. Physiol. Psychol. **84**, 488–495 (1973)
17. J. Gibbs, R.C. Young, G.P. Smith, Cholecystokinin elicits satiety in rats with open gastric fistulas. Nature **245**, 323–325 (1973)
18. T.R. Houpt, The sites of action of cholecystokinin in decreasing meal size in pigs. Physiol. Behav. **31**, 693–698 (1983)
19. S.M. Anika, T.R. Houpt, K.A. Houpt, Cholecystokinin and satiety in pigs. Am. J. Physiol. **240**, R310–317 (1981)
20. A.C. Clutter, R. Jiang, J.P. McCann, D.S. Buchanan, Plasma cholecystokinin-8 in pigs with divergent genetic potential for feed intake and growth. Domest. Anim. Endocrinol. **15**, 9–21 (1998)
21. J.C. Pekas, Effect of cholecystokinin immunization, enhanced food intake and growth of swine on lean yield and carcass composition. J. Nutr. **121**, 563–567 (1991)
22. J.C. Pekas, Immunogenicity of cholecystokinin octapeptide-conjugated antigens in pigs. J. Anim. Sci. **74**, 1953–1958 (1996)
23. E. Van Heugten, M.T. Coffey, J.M. Spears, Effects of immune challenge, dietary energy density, and source of energy on performance and immunity in weanling pigs. J. Anim. Sci. **74**, 2431–2440 (1996)
24. N.H. Stace, T.J. Palmer, S. Vaja, R.H. Dowling, Longterm pancreaticobiliary diversion stimulates hyperplastic and adenomatous nodules in the rat pancreas: A model for spontaneous tumor formation. Gut **28**, 265–268 (1987)
25. M.D. Barrachina, V. Martinez, L. Wang, J. Wei, Y. Tache, Synergistic interaction between leptin and cholecystokinin to reduce short term food intake in lean mice. Proc. Natl. Acad. Sci. **94**, 10455–10460 (1997)
26. M. Emond, G.J. Schwartz, E.E. Ladenheim, T.H. Moran, Central leptin modulates behavioral and neural responsivity to CCK. Am. J. Physiol. **276**, R1545–1549 (1999)
27. A.S. Kopin, W.F. Mathes, E.W. McBride, M. Nguyen, W. Al-Haider, F. Schmitz, et al., The cholecystokinin-A receptor mediates inhibition of food intake yet is not essential for the maintenance of body weight. J. Clin. Invest. **103**, 383–391 (1999)
28. J.J. Stern, C.A. Cudillo, J. Kruper, Ventromedial hypothalamus and short-term feeding suppression by caerulein in male rats. J. Comp. Physiol. Psychol. **90**, 484–490 (1976)
29. J. Glatzle, M.E. Kreis, K. Kawano, H.E. Raybould, T. Zittel, Postprandial neuronal activation in the nucleus of the solitary tract is partly mediated by CCK-A receptors. Am. J. Physiol. Regul. Intergr. Comp. Physiol. **281**, R222–229 (2001)
30. L.A. Eckel, T.A. Houpt, N. Geary, Estradiol treatment increases CCK-induced c-Fos expression in the brains of ovariectomized rats. Am. J. Physiol. **283**, R1378–1385 (2002)