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Effects of Ghrelin plasmid on growth performance and circulating GH, IGF-I, and SS in weaning piglets

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ABSTRACT

The aim of this study was to investigate effects of ghrelin plasmid (PCI-ghrelin-28aa) administration on plasma growth hormone (GH), insulin-like growth factor-I (IGF- I), and somatostatin (SS) levels, and growth performance in weaning pigs. Number I Junmu weaning piglets (8), 45-day-old, were randomly placed into 2 groups. Pigs in treatment groups were injected intra-muscularly with 1.5 mg kg^{-1} of ghrelin plasmid and in the control group, same dose of elution buffer. On day 0, 7, 21, 35, 49, and 63, body weight, food intake, and the circulating levels of GH, IGF-I, and SS were measured. The results showed that ghrelin plasmid significantly increased average daily weight gain (ADG) by 10.45% at 21 to 35 days. There were no significant differences between the treatment and control groups for daily food intake. Feed gain ratio (F/G) for the ghrelin treatment group was significantly better than control at 21–35d and 0–63d. Serum GH concentrations were significantly higher than control at 35 and 49 days. IGF-I levels were higher at 7 to 35 days in treated piglets and then decreased to a value which was significantly lower than those of the controls at 63 days. SS increased by 14% at 35 days and by 50% at 63 days. Results indicated that Ghrelin plasmid increased ADG and F/G, and regulated the levels of GH, IGF-I, and SS in piglets.

Key words: ADG, Ghrelin plasmid, GH, IGF-I, SS

Regulation of plasma growth hormone (GH), somatostatin (SS), and insulin-like growth factor-I (IGF- I) is essential for optimal growth and production. Ghrelin (GHS-R, Kojima *et al.*1999), important for growth hormone (GH) release (Peino *et al.* 2000), feeding behaviour (Kamegai *et al.* 2000), body weight gain (Wren *et al.* 2000, Toshinai *et al.* 2006), gastric functions (Dieguez *et al.* 2000) and endocrine regulation (Gil-Campos *et al.* 2006), is produced by enteroendocrine cells, A-like cells, oxyntic glands, and in the intestine, and is secreted into bloodstream (Kojima *et al.*1999, Wren *et al.* 2000, Toshinai *et al.* 2006). *In vivo*, i/v infusion of ghrelin in weaned pigs increased ghrelin, GH, insulin, and cortisol concentrations in serum, and decreased serum IGF-1 (Salfen *et al.*2004). Okumura *et al.* (2002) showed that ghrelin administration did not alter serum IGF-I. Ghrelin increased somatostatin mRNA levels in hypothalamus of GH-deficient rats (Seoane *et al.*2003). These findings suggested important links between ghrelin and other growth hormones. Wolff *et al.* (1990) reported that muscle cells were able to take up plasmid DNA injected intramuscularly and expressed the genes. Hence, many studies were focused on plasmid-mediated gene transfer thereafter. Plasmid DNA is considered simple to manipulate (Frederickson *et al.* 2003). Injection of exogenous GHRH DNA plasmid in mice and pigs stimulated growth (Draghia-Akli *et al.* 1997, 1999). In Taihu piglets, intramuscular injection of pGHRH gene plasmid stimulated GHRH, GH, and IGF-I excretion (Zhang *et al.* 2008). The pCI expression vector is widely used in plasmid-mediated gene transfer and is designed to promote constitutive expression of cloned DNA inserts in mammalian cells. We studied the effects of PCI-ghrelin-28aa on the performance and circulating levels of GH, SS, and IGF-I concentrations in weanling piglets.

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MATERIALS AND METHODS

Number I Junmu pigs (8), 45-day-old with average initial

weight of 16 kg were used in a 63-day study. Pigs were randomly assigned to 2 treatment groups, with 4 individuals per group, housed in individual pens. Food and water were offered *ad lib*. Corn and soybean meal based diets meet nutrient recommendation for pig by NRC (1998). A 2-phase feeding programme, from 45–65 days of age (DE: 13.89 MJ/kg, CP: 19.73%) and from 66–107 days of age (DE: 13.18 MJ/kg, CP: 16.47%) was adopted.

Ghrelin plasmid: The plasmid containing the ghrelin gene was constructed by our team (Yang *et al.*2006). The recombination plasmid was identified by PCR, digestion with internal restriction enzymes, and sequencing. After showing successful expression of the recombinant ghrelin in the cells, the plasmid DNA construct was transfected into *E. coli*.

Plasmids were extracted and purified with the endotoxin-free plasmid mini kit and dissolved in elution buffer to a final concentration of 1–2 mg ml^{-1}. Plasmid purity and concentration were confirmed by 1% agarose gel electrophoresis and UV absorption at 260 and 280 nm. The percentage of supercoiled DNA and the OD 260/280 ratio of these preparations were approximately >92–96% and 1.7–1.9, respectively.

On the first day, pigs were injected with a high osmotic pressure sucrose solution (0.2 ml kg^{-1} BW) as an immunologic adjuvant; 20 min later, the treatment group was treated with recombinant plasmid PCI-Ghrelin-28aa (1.5 mg kg^{-1} in 16 ml, dissolved in elution buffer) by intramuscular injection into the semitendinosus muscle at a depth of 1 cm, whereas, the control group received a similar volume of elution buffer.

Test index and method: Pigs were weighed before injection, and at 7, 21, 35, 49, and 63 days thereafter. Blood samples were collected through the precaval vein on the day of injection, as well as 7, 21, 35, 49, and 63 days after injection. Blood samples were divided into 2 portions. One portion was prepared for serum by inversion and incubation at 37°C for 30–60 min, followed by incubation at 4°C overnight to allow the clot to contract. The serum was decanted away from the clot into a new centrifuge tube and spun at 1300×g for 15 min to remove other materials. The other portion was used for separation of plasma where collected blood samples containing EDTA-aprotinin (12.5 µl ml^{-1} of blood) were centrifuged at 1300×g for 10 min at 4°C. Separated plasma and serum were stored at –70°C until analysis.

Food intake was recorded daily. Average daily gain (ADG) and food gain ratio F/G were calculated for the periods of 0 to 7, 7 to 21, 21 to 35, 35 to 49, and 49 to 63 days. GH and IGF-I concentrations were measured by antibody radioimmunoassay (RIA) after extraction of serum (GH and IGF-I radioimmunoassay kit). The SS concentration was measured by RIA after extraction of plasma (SS radioimmunoassay kit).

Statistical analysis: Collected data were analyzed using SPSS 13.0 software and were presented as mean±SE. The significance test was analyzed by the independent-samples T-test. Differences were considered highly significant at $P<0.01$ and significant at $P<0.05$.

RESULTS AND DISCUSSION

Growth performance: All of the pigs were healthy and grew well throughout the experiment. The effects of ghrelin plasmid on growth performance of pigs are given in Table 1.

The results indicated that the ghrelin gene plasmid increased ADG of pigs in injected group on days 7 to 21, 21 to 35 and 0 to 63, and was 10.45% higher on days 21 to 35 in treated group than the control ($P<0.05$). After day 35, ADG of treatment group was lower than the control, but there were no significant differences. Likewise, there were no significant differences between the treatment and control groups for daily feed intake ($P>0.05$). During the experiment period, there were no significant differences between the treatment and control groups ($P>0.05$) for feed gain ratio, except for days 21 to 35 and 0 to 63, which were significantly lower than control ($P<0.05$).

Blood concentrations of GH, IGF-I, SS: The results showed that the serum GH concentrations in subjects receiving ghrelin treatment tended to increase from the first postoperative day and were significantly higher ($P<0.01$) than those in the control groups at 35 and 49 days (Fig. 1). Similarly, SS levels (Fig. 2) were significantly higher than in the control group at 35 and 63 days (by 14%, $P<0.05$ and 50%, $P<0.01$, respectively). Before 35 days, IGF-I levels (Fig. 3) showed the same rising trend, with no notable differences; then began to fall, and were significantly lower ($P<0.05$) than those of the controls at 63 days.

Table 1. Growth performance of weaning pigs after the infusion of ghrelin gene plasmid or elution buffer

Growth stage(d)	Groups	0–7	7–21	21–35	35–49	49–63	0–63
Daily feed intake (kg d^{-1})	Ghrelin	0.71±0.01	0.98±0.01	1.17±0.01	1.49±0.03	1.54±0.06	1.316±0.03
	Control	0.72±0.02	1.01±0.03	1.07±0.03	1.42±0.03	1.61±0.02	1.321±0.02
Daily gain (g d^{-1})	Ghrelin	0.37±0.01	0.60±0.03	0.74±0.03^{b}	0.73±0.02	0.87±0.04	0.667±0.03
	Control	0.38±0.03	0.54±0.05	0.67±0.02^{a}	0.74±0.04	0.92±0.01	0.639±0.04
Feed gain ratio (F/G)	Ghrelin	1.90±0.10	1.69±0.24	1.49±0.11^{a}	1.75±0.03	1.68±0.04	2.023±0.10^{a}
	Control	1.95±0.20	1.82±0.13	1.63±0.06^{b}	1.73±0.07	1.71±0.04	2.117±0.08^{b}

Different superscripts in the same line of the same stage indicate significant differences ($P<0.05$).

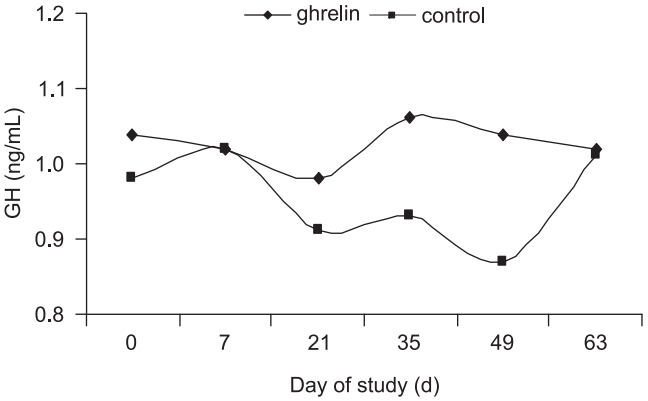

Fig. 1. Blood concentrations of GH after the infusion of ghrelin gene plasmid or elution buffer.

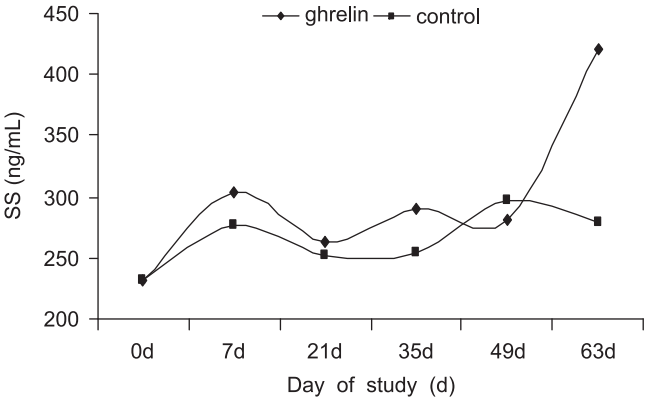

Fig. 2. Blood concentrations of SS after the infusion of ghrelin gene plasmid or elution buffer.

Plasmid DNA is considered safe, economical, and simple to manipulate (Frederickson *et al.* 2003), obviating the necessity to construct viral vectors. Further, the transfer procedure may be repeated easily because naked plasmid DNA has little antigenicity to the host body (Gonin and Gaillard 2004). Skeletal muscle fibers consist of long, multinucleated cells, have a slow turnover rate, and the nuclei of mature muscle cells do not undergo division; therefore, gene expression is stable in muscle cells (Hagstrom *et al.*1996), leading to the assumption that ghrelin plasmid may be a suitable approach for this tissue.

Weaning transition is a critical period in the life cycle of a pig. In previous research, weaned pigs were intravenously infused with human ghrelin (3 times daily for 5 days), which induced positive weight gain and increased feeding duration compared to saline-infused controls (Salfen *et al.* 2004). Exogenous ghrelin infused via the marginal ear vein @ 1 g d^{-1} per pig could cause a variety of behavioral effects, but no significant influence was found on average ADG and average daily feed intake (ADFI) in weanling piglets (P>0.05) (Wu *et al.* 2008). In this study, after weaning pigs were fed for 14-day as adjustment period to reduce the effects of stress on weight and hormone secretions. The average weight gain

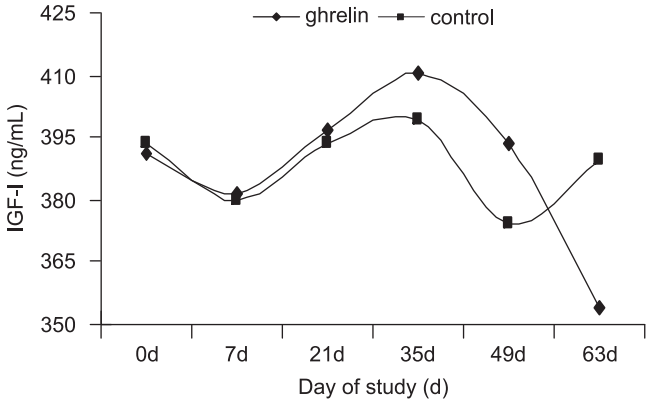

Fig. 3. Blood concentrations of IGF-I after the infusion of ghrelin gene plasmid or elution buffer.

of experimental piglets was significantly higher (P<0.05) on postoperative days 21–35, then showing lower gain than the control. Feed gain ratio (F/G) for the ghrelin treatment group was significantly better (P<0.05) than control at 21–35d and 0–63d. These results showed that plasmid PCI-ghrelin-28aa has a similar function to ghrelin; however, ghrelin plasmid cannot keep expressing steadily for a longer period of time. When ghrelin plasmid was degraded, or exogenous ghrelin was decreased, other hormones maintained the performance of piglets.

GH is the principal hormone involved in somatic growth and body composition. Ghrelin, an endogenous ligand for growth hormone secretagogue receptor (GHS-Rs), acted together with SS and IGF-I to regulate the secretion of GH. Ghrelin displayed strong GH-releasing activity. In the present experiment, ghrelin enhanced serum GH level, peaked at 35 days after injection, and continued at higher level until 49 days, then slowly decreased to the same level of the control group until 63 days.

The elevation in GH levels was not unexpected, especially in consideration that ghrelin is a potent stimulator of GH release in rats, and on a pharmacological level, may be even more efficacious than GHRH (Kojima *et al.* 1999). Ghrelin stimulated GH release both *in vitro* and *in vivo* in a dose-dependent manner (Peino *et al.* 2000). The higher level of GH was only maintained for 15 days and might be correlated to the increase of SS and IGF-I levels, which play a role of negative feedback regulation. This would offset the promoting effect of the expression of ghrelin plasmid in muscle.

SS significantly increased by 14 and 50% at 35 and 63 days, respectively. The SS level increased at 35 days and could have inhibited the secretion of GH, possibly explaining the reason of no difference in pig performance between the treatment and control groups after 35 days, as well as the reason for decreased GH level after 49 days.

IGF-I levels were higher at 7 to 35 days in treated piglets, and then decreased compared to the control, with significant differences (P<0.05) at 63 days. Previous observations showed that when weaned pigs were initially infused with

human ghrelin (2 μg kg^{-1} BW) 3 times daily for 5 days, serum IGF-I levels initially declined in both treatment groups from days 1 to 2 ($P < 0.05$), but then increased ($P< 0.05$) from days 5 to 8 (Salfen *et al.* 2004). Another study indicated that serum IGF-I levels were not altered by ghrelin administration. GH and SS levels rose significantly from 35 days, showing that ghrelin plasmid was effective. While SS levels showed significant changes until 63 days, we speculated that either (i) the effect of ghrelin on IGF-I secretion begun at 63 days, or (ii) that ghrelin might have little effect on serum IGF-I levels, because the IGF-I level significantly decreased at 63 days ($P<0.05$), perhaps due to feedback regulation of GH.

All of our results showed that function of plasmid PCI-ghrelin-28aa is similar to ghrelin. Further study is essential to determine the optimal dosage of ghrelin plasmid for injection and timing of injection, to improve techniques such as hydrodynamic methods and electroporation to improve muscle transfection.

In conclusion, ghrelin plasmid injection @ 1.5 mg kg^{-1} can stimulate GH release and increase body weight gain and circulating SS levels, along with decreasing IGF-I levels. GH and IGF-I levels were increased, with the high GH levels promoting SS levels, and high SS level also suppressing GH and IGF-I.

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REFERENCE

Dieguez C and Casanueva F F. 2000. Ghrelin: A step forward in the understanding of somatotroph cell function and growth regulation. *European Journal of Endocrinology / European Federation of Endocrine Societies* **142**: 413–17.

Draghia-Akli R, Fiorotto M L and Hill L A. 1999. Nonclinical toxicology in support of licensure of gene therapies. Myogenic expression of an injectable protease resistant growth hormone-releasing hormone augments long term growth in pigs. *Nature Biotechnology* **17**: 1179–83.

Draghia-Akli R, Li X G and Schwartz RJ. 1997. Enhanced growth by ectopic expression of growth hormone releasing hormone using an injectable myogenic vector. *Nature Biotechnology* **15**: 1285–89.

Frederickson R M, Carter B J and Pilaro A M. 2003. Nonclinical toxicology in support of licensure of gene therapies. *Molecular Therapy: Journal of the American Society of Gene Therapy* **8**: 8–10.

Gil-Campos M, Aguilera C M, Cañete R and Gil A. 2006. Ghrelin: a hormone regulating food intake and energy homeostasis. *British Journal of Nutrition* **96**: 201–26.

Gonin P and Gaillard C.2004.Gene transfer vector biodistribution: pivotal safety studies in clinical gene therapy development. *Gene Therapy* **11**: S98– 08.

Hagstrom J E, Rybakova I N, Staeva T, Wolff J A and Ervasti J M**.** 1996. Nonnuclear DNA binding proteins in striated muscle. *Biochemical and Molecular Medicine* **58**: 113–21.

Kamegai J, Tamura H, Shimizu T, Ishii S, Sugihara H and Wakabayashi I. 2000. Central effect of ghrelin, an endogenous growth hormone secretagogue, on hypothalamic peptide gene expression. *Endocrinology* **141**: 4797–4800.

Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H and Kangawa K. 1999. Ghrelin is a novel growth-hormone-releasing acylated peptide from stomach. *Nature* **402**: 656– 60.

Maura Arosio, Cristina L. Ronchi, Carlotta Gebbia, Vincenzo Cappiello, Paolo Beck-Peccoz and Maddalena Peracchi. 2003. Stimulatory effects of ghrelin on circulating somatostatin and pancreatic polypeptide levels. *Journal of Clinical Endocrinology and Metabolism* **88**: 701–04.

Okumura H, Nagaya N, Enomoto M, Nakagawa E, Oya H and Kangawa K. 2002. vasodilatory effect of ghrelin, an endogenous peptide from the stomach. *Journal of Cardiovascular Pharmacology* **39**: 779–83.

Peino R, Baldelli R, Rodriguez-Garcia J, Rodriguez-Segade S, Kojima M, Kangawa K, Arvat E, Ghigo E, Dieguez C and Casanueva F F. 2000. Ghrelin-induced growth hormone secretion in humans. *European Journal of Endocrinology / European Federation of Endocrine Societies* **143**(6): R11–14.

Salfen B E, Carroll J A, Keisler D H and Strauch T A. 2004. Effects of exogenous ghrelin on feed intake, weight gain, behavior, and endocrine responses in weanling pigs. *Journal of Animal Science* **82**: 1957–66.

Seoane LM, Lopez M, Tovar S, Casanueva F F, Senaris R and Dieguez C. 2003. Agouti-related peptide, neuropeptide Y, and somatostatin-producing neurons are targets for ghrelin actions in the rat hypothalamus. *Endocrinology* **144**: 544–51.

Toshinai K, Yamaguchi H, Sun Y, Smith RG, Yamanaka A, Sakurai T, Date Y, Mondal M S, Shimbara T, Kawagoe T, Murakami N, Miyazato M, Kangawa K and Nakazato M. 2006. Des-acyl ghrelin induces food intake by a mechanism independent of the growth hormone secretagogue receptor. *Endocrinology* **147**: 2306–14.

Wolff J A, Malone R W, Williams P, Chong W, Acsadi G, Jani A and Felgner P L. 1990. Direct gene transfer into mouse muscle *in vivo*. *Science* **247**: 1465–68.

Wren A M, Small C J, Ward H L, Murphy K G, Dakin C L, Taheri S, Kennedy A R, Roberts G H, Morgan D G, Ghatei M A and Bloom S R. 2000. The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. *Endocrinology* **141**: 4325–28.

Wu Xing-li , Tang Mao-yan , Ma Qiu-gang , Hu Xinxu and Ji Cheng. 2008. Effects of exogenous ghrelin on the behaviors and performance of weanling piglets. *Asian-Australasian Journal of Animal Sciences* **21**: 861–67.

Yang Wen-yan, Yang Lian-yu and Geng Chun-yin. 2006. construction of eukaryotic expression recombinant plasmid of swine ghrelin gene and histogenic immunity examination in mice. *Journal of Jaha Agricultural University* **28**: 667–70. (In Chinese.)

Zhang Yong , Yu-Jing Zhu , Zhe Li , Zhou An-Guo, Zhang Jing-Hai, Bardsley R G and Gibson S. 2008. Injection of porcine growth hormone releasing hormone gene plasmid in skeletal muscle increases piglets' growth and whole body protein turnover. *Livestock Science* **115**: 279–86.