



Effects of Feed Restriction and Fermented Potato Protein Supplementation on *IGF1*, *GHR* and *Kiss1* mRNA Expression in Intact Nursery Gilts

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ABSTRACT

Background: Decreased feed intake is a common stress response presented after weaning that leads to a negative effect on performances. Health management and feed supplementation were commonly used to solve such problems. The objectives of this research were to study the effects of nutritional stress and the benefit of fermented potato protein supplementation in intact nursery gilts.

Methods: Prepubertal nursery gilts were assigned into the following feed treatments for 30 days: 1) normal feed, 2) normal feed with fermented potato protein (FPP) supplementation, 3) restricted feed which received 60% amount of normal feed and 4) restricted feed with FPP supplementation.

Result: Serum IGF-1 and *IGF1* mRNA expression of semitendinosus muscle were significantly increased by FPP supplementation ($P < 0.05$). All pig groups showed no different on serum cortisol and hypothalamic *Kiss1* mRNA expression levels. The degree of feed restriction was suggested to be an important determinant of response of serum IGF-1, cortisol and hypothalamic *Kiss1* mRNA expression. In this study, an elevated serum IGF-1 concentration correlated with *IGF1* mRNA upregulation in a muscle rather than in a liver.

Key words: Growth hormone receptor, IGF-1, Kisspeptin, Metabolic stress, Nursery pig.

INTRODUCTION

Weaned pigs have to go through changes of diet, barn condition and mixing with other pigs in new pens. These stressors lead to a reduced growth performance, feed intake reduction, maldigestion and absorption (Lallès *et al.*, 2004) which can result in economic losses. Many corrective measures, such as raising management (Mei and Gan 2018), nutritional management and feed supplementation (Namted *et al.*, 2020) were studied. Previous studies on fermented potato protein (FPP) supplementation in suckling pigs were showed to increase serum IGF-1 level (Poltep *et al.*, 2016), decrease mortality rate at 24 h of age (Kummer *et al.*, 2015) and increase *IGF1* expression in peripheral white blood cell (Poltep *et al.*, 2016).

Circulatory IGF-1 concentration in swine demonstrated a correlation with growth (Owens *et al.*, 1999), stress and current nutritional statuses such as feed restriction and fasting (Thorson *et al.*, 2018). However, the lack of circulatory IGF-1 in rodents did not affect body growth and organ size, except only smaller kidney size (Sjögren *et al.*, 1999). Nevertheless, intravenous administration of IGF-1 was able to increase growth in hypophysectomized rats (Schoenle *et al.*, 1982). The importance of the autocrine/paracrine function of IGF-1 can be understood from the fact that local instead of peripheral administration of IGF-1 results in muscle hypertrophy (Adams and McCue, 1998).

In swine, there are two populations of kisspeptin neurons in hypothalamus periventricular nucleus (PeN) and arcuate nucleus (ARC) (Tomikawa *et al.*, 2010), but unlike other species, the ARC in swine expresses kisspeptin from the early age then continues constantly until the onset of

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puberty (Ieda *et al.*, 2014). Previous studies on the effects of feed restriction showed decreased ARC *Kiss1* mRNA expression in intact adult female rats (Roa *et al.*, 2009) and decreased hypothalamic *Kiss1* mRNA expression of the prepubertal ewe (Wang *et al.*, 2016). In addition, peripheral administration of IGF-1 upregulated anteroventral periventricular nucleus (AVPV) kisspeptin neuron population of the hypothalamus (Hiney *et al.*, 2009).

Currently, there is a lack of data evidencing the effect of feed restriction and feed supplementation on growth parameters and ARC kisspeptin expression of pigs. The objectives of this study were to determine the effect of nutritional stress and the benefit of FPP as a supplementation in intact nursery gilts. It is hypothesized that feed restriction decreases growth rate and *Kiss1* mRNA expression during the prepubertal period and FPP supplementation revives growth rate, circulatory IGF-1 and *Kiss1* mRNA expression.

MATERIALS AND METHODS

This study was approved by Animal Care and Use for the scientific research committee, Kasetsart University, Thailand (Approval number ACKU 59-vet-001). The research was conducted from February 2019 to March 2020 at the Faculty of Veterinary Medicine Kasetsart University Kamphaeng Saen campus. Twenty crossbreds (Landrace × Large white × Duroc) gilts at the age of 5 weeks old were randomly assigned into four groups of 5 pigs per dietary treatment. The treatments were: normal feed (NF), normal feed with FPP supplementation (NF+S), restricted feed with 60% of normal feed given (RF) and restricted feed with FPP supplementation (RF+S). Pigs were fed with 2 phases of feed formulations with phase 1 diet started from day-7 (5-weeks-old piglets) to day 5. Then phase 2 diet were given afterward until the end of study (Table 1). The FPP supplementation was used in phase 2 feed formulation at 500 g per ton of feed. From experimental day-7 to 0, animals were fed *ad libitum*. From day 1 to day 30, NF and NF+S groups were fed according to standard nutrients requirement of swine (NRC, 2012), feed restriction treatments were applied in RF and RF+S groups.

Pigs were weighed every 5 days from day 0 to day 30 of the experiment. Serum samples were collected at day 0, 10, 21 and 30. On day 31, the pigs were euthanized. Tissue samples from livers, semitendinosus muscles and hypothalami were collected and stored at -80°C with in 15 min until RNA extraction.

The serum concentration of IGF-1 was evaluated using the ELISA test (commercial ELISA kit, Biomatik, Canada). The serum concentration of cortisol was analyzed from serum collected on day 30 by using the EIA method (Brown *et al.*, 2004).

The RNA extraction was done using Direct-zol RNA MiniPrep kit (Zymo Research, USA). Reverse transcription was performed using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., USA). The cDNA synthesis was performed by SimpliAmp Thermal Cycler (Thermo Fisher Scientific Inc., USA). The thermocycling threshold was 42°C for 60 min followed by 70°C for 5 min. All cDNA were kept under -20°C for future laboratory analysis.

The primers were newly designed or referred from previous publications, R^2 and efficiency were obtained by mixed samples cDNA, as shown in Table 2. The qPCR reactions were run in CFX96 Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA). All samples were performed in triplicate. The thermocycling threshold was 1 cycle of 98°C for 5 min, followed by 40 cycles of 98°C for 30 s, 60°C for 30 s and 72°C for 30 s. Melting temperature analysis was conducted between 65°C and 95°C using a 30 min ramp time and continuous fluorescence detection to determine primer specificity for each reaction. The housekeeping gene beta-actin (*ACTB*) was selected as reference to normalize the variance in amounts of cDNA input in the reactions. The normalized expression of the target gene was calculated by

$\Delta\Delta Cq$ method using Bio-Rad CFX Mastero 1.1 (version 4.1.2433.12219). The qPCR products of each primer pair were submitted for sequencing at 1st BASE Laboratories, SDN BHD, Malaysia.

Statistical analysis was performed using SPSS version 24 (IBM, USA). Treatment differences in body weight and concentrations of IGF-1 hormone were compared by two-way repeated measurement analysis of variance (ANOVA) with feed regimen and FPP supplementation as main effects and time as within-subject effect. The normalized expression of *IGF1* mRNA and *GHR* mRNA of the liver and *IGF1* mRNA of the muscle were compared between experimental groups by two-way ANOVA with feed regimen and FPP supplementation as main effects. Serum cortisol, *Kiss1* mRNA expression of the hypothalamus and *GHR* mRNA of the muscle were compared between treatments by nonparametric Kruskal-Wallis test, due to the non-equality of error variances. The difference was considered statistically significant ($P < 0.05$).

RESULTS AND DISCUSSION

The lower body weight (Fig 1) and ADG (Table 3) in restricted feed groups ($P < 0.001$) indicated that the level of feed restriction in the present study directly influenced the body weight and ADG. The FPP supplementation did not

Table 1: Diet composition and nutritional profiles of the two feed formulas.

Item	Phase 1 diet	Phase 2 diet
Proximate analysis, %		
Crude protein	21.77	19.97
Crude fat	6.01	8.75
Crude fiber	1.58	2.10
Calcium	0.71	0.70
Phosphorus	0.68	0.69
Metabolizable energy (kcal/kg)	3658.33	3566.78
Ingredients, g/kg		
Broken rice	510	600
Palm oil	10	50
Soybean meal	50	50
Steamed beans meal	-	160
L-Lysine	2.5	3.5
DL-Methionine	2.5	2
L-Threonine	1.5	2
Monocalcium phosphate	11	18
Limestone	9	8
Salt	1	4
Vitamin and mineral premix [†]	2.5	2.5
Soy protein concentrate	100	100
Milk	200	-
High quality protein	100	-

[†]Supplied per kilogram diet: Vitamin A 18,000 IU; Vitamin D3 3,600 IU; Vitamin E 25 IU; Vitamin K 3.5 mg; Vitamin B1 1 mg; Vitamin B2 8.8 mg; Vitamin B12 0.14 mg; Niacin 7 mg; Pantothenic 18 mg; folic acid 0.5 mg; Cu 130 mg, Fe 150 mg, Mn 50 mg, Zn 130 mg, I 0.25 mg, Se 0.3 mg.

compensate the body weight and ADG for the loss of feed intake. These similar findings were documented in a previous study (Poltep *et al.*, 2016).

The serum IGF-1 of pigs in NF+S and RF+S that received FPP was significantly higher than NF and RF groups which were raised without FPP (Fig 2A). Reports from several studies showed that the relation between feed restriction and IGF-1 levels are still inconclusive. Combes *et al.* (1997) found that reductions in serum IGF-1 levels in response to feed restriction was inconsistent throughout swine development. The serum IGF-1 was not significantly different in feed restricted swine at the body weight of 40 kg and during 100-130 kg, but showed statistically reduction when measured in 70 kg swine. In contrast, a study showed that feed restriction in swine reduced serum IGF-1 (Thorson *et al.*, 2018). The rise in IGF-1 from FPP supplementation were also found in a previous study (Poltep *et al.*, 2016).

The serum concentrations of cortisol in all groups were not significantly different regardless of feed regimen or FPP addition for 30 days (Fig 2B). It has been reported that serum concentration of cortisol in swine experiencing feed restriction of less than 21 days underwent no change in their serum cortisol level (Prunier *et al.*, 1993; Thorson *et al.*, 2018). However, feed restriction for 21 days in 78-98 days old pigs led to an increase in serum cortisol (Metges *et al.*, 2015). The variations of cortisol responses to feed restriction between different studies may be from differences between age, degree of feed restriction and duration of the treatment of each study. The results of this

study indicated that restricted feed alone had no effect on serum IGF-1 and cortisol concentrations and the FPP supplementation raised the IGF-1 levels but not serum cortisol concentration.

The *IGF1* mRNA expression from semitendinosus muscle was significantly higher ($P=0.019$) in NF+S and RF+S than that of NF and RF groups (Fig 3A). The *GHR* mRNA levels of semitendinosus muscle (Fig 3B) were not different in all groups. There was also no difference in the *IGF1* and *GHR* mRNA expression levels in the liver (Fig 3C and 3D) between all groups, although FPP supplementation had shown to increase *IGF1* gene expression in the liver of broiler chickens (Lertpimonpan *et al.*, 2019). The liver is known to be the principal source of circulatory IGF-1 (Sjögren *et al.*, 1999), but in young pigs, possibly major source of serum IGF-1 is from non-hepatic tissue such as skeletal muscle (Lee *et al.*, 1993). In results from this study indicated that the increase in serum concentration of IGF-1 was resulted from non-hepatic tissue production and the higher *IGF1* mRNA expression in the semitendinosus muscle correlated with the increase level of serum IGF-1.

As *Kiss1* gene expression in the ARC of hypothalamus were not significantly different in all animal groups (Fig 3E), hence, the results from this study indicated that increased serum IGF-1 by the FPP supplementation did not alter *Kiss1* mRNA expression in the hypothalamus of prepubertal pigs. Similar findings found that IGF-1 stimulated the AVPV kisspeptin expression but had a negligible effect on ARC kisspeptin neuron population of mice (Hiney *et al.*, 2009). This resemble the findings by Thorson *et al.* (2018), where

Table 2: Primers sequence and other details.

Gene	Primers	Gene bank accession no.	Amplification length, BP	T _a , °C	E [†]	Reference
<i>GHR</i>	Forward: CAGTGGAAATGATGGACC Reverse: GGAAACCGGAAATCTTCTTCACATGC	NM_214254.2	191	60	95.8	Designed in this study.
<i>IGF1</i>	Forward: TCTTCTACTTGGCCCTGTGCTT Reverse: TGGGCTTGTTGAAATAAAAGCC	NM_214256.1	136	60	91.8	(Slifierz <i>et al.</i> , 2013) Designed in this study.
<i>Kiss1</i>	Forward: GGAGACGTCATCTGGCTTTT Reverse: CGGGCCTGTAGATCTAGGATT	AB466320.16	301	60	103	(Thorson <i>et al.</i> , 2018)
<i>ACTB</i>	Forward: GGCCGCACCACTGGCATTGTCAT Reverse: AGGTCCAGACGCAGGATGGCG	DQ845171.1	104	60	99.5	(Zhuo <i>et al.</i> , 2014)

Growth hormone receptor (*GHR*), insulin-like growth factor 1 (*IGF1*), kisspeptin (*Kiss1*) and beta-actin (*ACTB*).

Every primer pair have R²>0.980.

[†]Efficiency.

Table 3: Average daily weight gain (ADG) of the nursery gilts.

ADG	Supplementation		Average by feed regimen
	No supplement	Supplemented with FPP (s)	
Feed regimen			
Normal feed (NF)	403.49±11.80	412.16±7.06	407.83±9.77 ^a
Restricted feed (RF)	244.24±4.07	243.48±9.31	243.85±7.18 ^b
Average by supplementation	323.87±19.87	327.82±20.59	

Average daily gain (ADG; g/d) ±SE. ^{a,b} the values that do not share the same letter in the superscript are statistically significant, $P<0.01$.

short-term (10 d) feed restriction with mild body weight loss in ovariectomized late prepubertal gilts had no changes in the number of cells that express *Kiss1* in the medial arcuate nucleus of hypothalamus and also had no differences in serum cortisol level, even though serum IGF-1 were lowered. Contrarily, Zhou *et al.* (2014) observed that long-term feed

restriction (100 d) with body weight loss in intact pubertal gilts decreased hypothalamic *Kiss1* mRNA expression. Suggested that transcription of hypothalamic kisspeptin appeared to be influenced by magnitude and duration of feed restriction as reviewed by Lents (2019). Therefore, feed restriction procedure, cortisol or combination of these factors

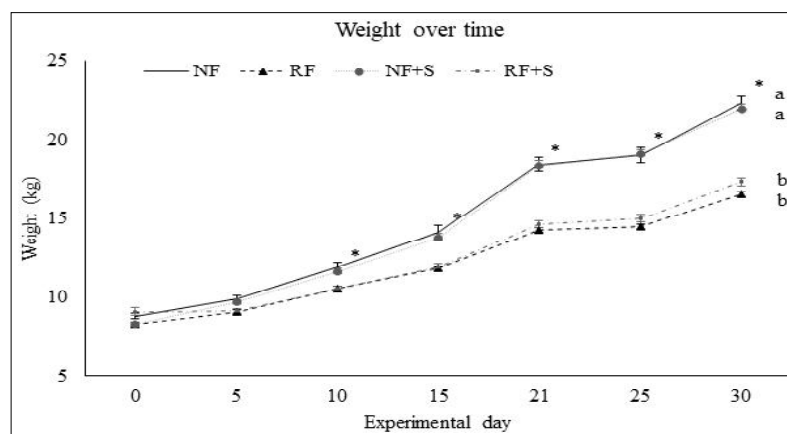


Fig 1: Mean $\pm$ SE of body weight of nursery gilts in each treatments. Normal feed (NF), normal feed with FPP (NF+S), restricted feed (RF) and restricted feed with FPP (RF + S). *Feed regimen $\times$ time interaction, $P < 0.05$. ^{a,b} the values that do not share the same letter in the superscript are statistically significant, $P < 0.05$.

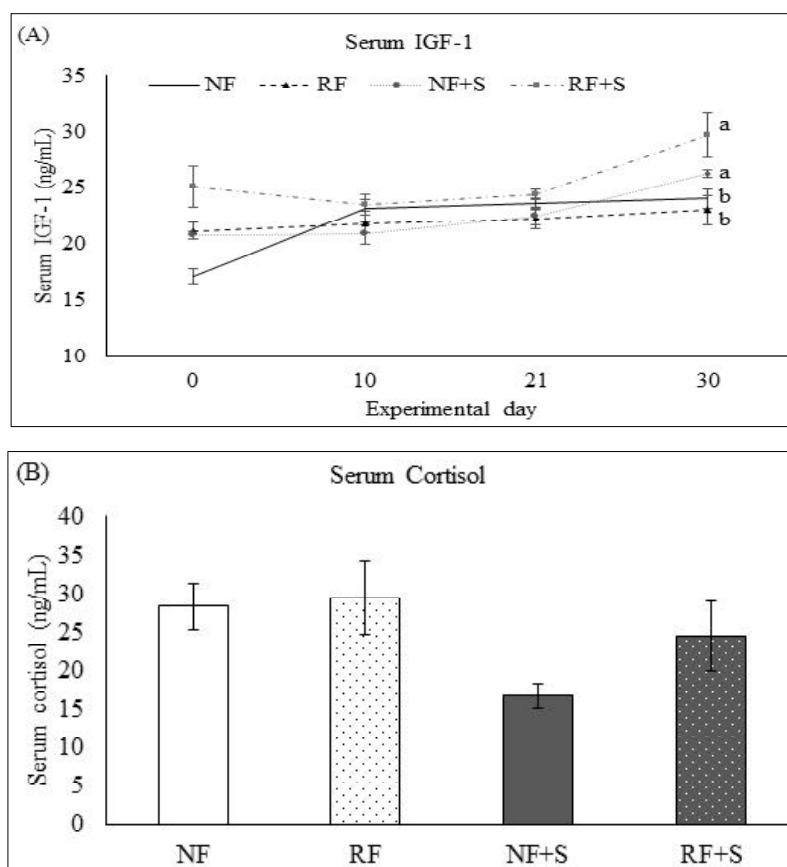


Fig 2: Mean $\pm$ SE of (A) concentration of IGF-1 in serum and (B) concentration of cortisol in the serum of nursery gilts that were treated with normal feed (NF), normal feed with FPP (NF+S), restricted feed (RF), restricted feed with FPP (RF+S). ^{a,b} the values that do not share the same letter in the superscript are statistically significant, $P < 0.05$.

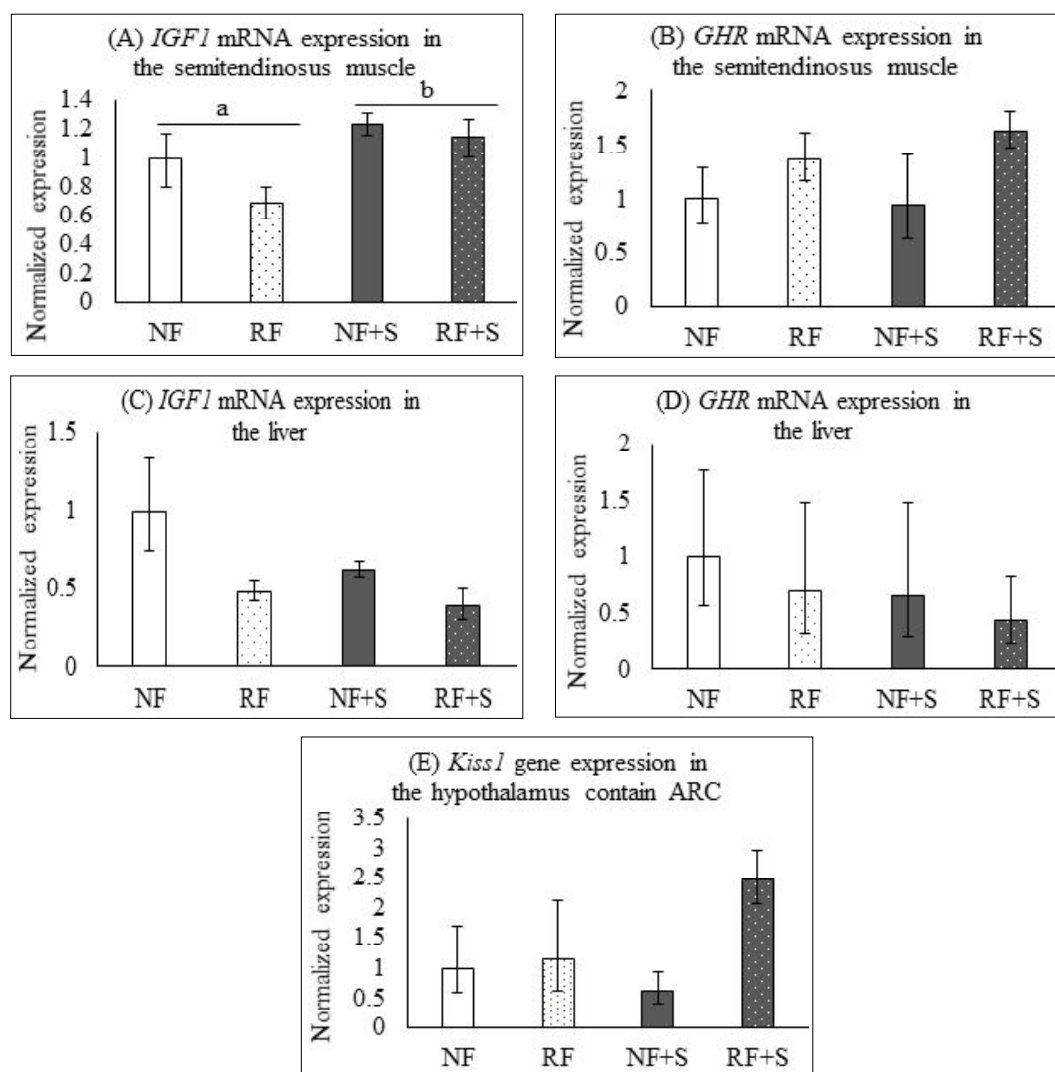


Fig 3: The geometric mean of the normalized expression $\pm 2(\text{exp. (lg)} \pm \text{SEM of exp. (lg)})$ of nursery gilts that were treated with normal feed (NF), normal feed with FPP (NF+S), restricted feed (RF), restricted feed with FPP (RF+S). Where (lg) is $\log_2$. ^{a,b} the values that do not share the same letter in the superscript are statistically significant, $P < 0.05$.

may influence the response of the *Kiss1* mRNA expression in swine.

CONCLUSION

FPP supplementation significantly increased serum IGF-1 and *IGF1* mRNA expression levels of semitendinosus muscle. The increased serum IGF-1 is produced by non-hepatic tissue. No effect of feed restriction on hypothalamic *Kiss1* mRNA expression and serum cortisol of intact prepubertal nursery gilts were observed.

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Conflict of interest: None.

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