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UNDER STARVED CONDITIONS THE EXPRESSION PATTERN OF HYPOTHALAMIC KISSPEPTIN/GPR54 AND ROLE OF KISSPEPTIN-10 IN RESTORATION OF LH SECRETION IN PREPUBERTAL EWES

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ABSTRACT

Kisspeptin and its receptor G protein-coupled receptor (GPR54) have been shown to play a crucial role in regulation of GnRH secretion. The objective of this study was to evaluate the role of Kisspeptin/GPR54 in inhibition of LH secretion of prepubertal ewe sunder feed restricted conditions. Three months old, female, Small Tail Han sheep, around 16.2 kg body weights were used in this study. During experiment I, in control group, ewes were fed with standard diet (n=4), while in feed restricted group, ewes were subjected to 40% restriction in daily feed intake (n=4). Thirty days later, blood serum and hypothalamus of all the animals were obtained to determine secretion level of LH, insulin, leptin and expression of *KiSS-1* and *GPR54*. The results showed that feed restriction significantly decreased hypothalamic expression of *KiSS-1* ($P<0.01$) and serum concentration of LH ($P<0.05$), insulin ($P<0.05$) and leptin ($P<0.01$) in prepubertal ewes. In experiment II, the same animals underfeed restriction were injected with 1mg of kisspeptin-10 (n=4; treatment group), and normal saline (n=4; control group). Serial blood samples were collected at 15-min intervals for 180 min to analyze the response curve of LH secretion after injection. The results showed that in prepubertal ewes under feed restriction injection with kisspeptin-10 significantly increased the LH secretion ($P<0.01$). The results indicate that the inhibitory effect of long-term feed restriction on LH secretion in prepubertal ewes is possibly because of a decrease in hypothalamic expression of *KiSS-1*.

Key words: Feed restriction, LH, Kisspeptin/GPR54Prepubertal ewes.

INTRODUCTION

It is well known that nutrition is closely linked to puberty onset in domestic animal. The nutritional limitations results in delayed puberty by restricted luteinizing hormone (LH) secretions. (Prunier and Quesnel, 2000; Blache *et al*. 2007). It has been evaluated that the inhibition of LH secretion is because of decreased gonadotropin hormone (GnRH) release from hypothalamus, rather than by inhibition of pituitary gonadal axis sensitivity to GnRH(Kile *et al*. 1991; I'Anson *et al*. 2000). Although it is well established that feed restriction delays puberty onset by suppressing GnRH/LH secretion, but still the exact molecular mechanism is not clear.

A wide array of central players in the inhibitory and stimulatory controls of GnRH neuron has been identified (Terasawa 2005; Todman *et al*. 2005; Christensen *et al*. 2012). It is likely that feed restriction results in suppressed GnRH/LH secretion by regulating the inhibitory or stimulatory controls of GnRH neurons. Recently, Kisspeptin/GPR54 has been identified as the key molecular channel for regulating reproductive endocrinology by a series of pharmacological and genetic studies (Papaoiconomou *et al*. 2011). *KiSS-1*gene encodes a number of structurally-related peptides (kisspeptin-54, kisspeptin-14, kisspeptin-13 and kisspeptin-10), which have a similar function by binding to their receptor GPR54. More recently, studies in rodents have shown that Kisspeptin/GPR54 involves in regulation of GnRH secretion by gonadal steroids, photoperiod and nutrition (Revel *et al*. 2007; Papaoiconomou *et al*. 2011; Dedes 2012).There is limited information available in literature about the role of hypothalamic Kisspeptin/GPR54 in suppression of LH secretion by feed restriction. Therefore, it is hypothesized that feed restriction may target directly the hypothalamic Kisspeptin/GPR54 to suppress LH secretion in prepubertal ewes. To test this hypothesis, expression of hypothalamic Kisspeptin/GPR54 and the effect of kisspeptin-10 on LH secretion in prepubertal ewes under long-term feed restriction were analyzed.

MATERIALS AND METHODS

Animals and drugs: Sixteen female Small Tail Han Sheep (native breed), around 16.2 kg body weight and three months of age, were used in this study. Ethical approval for the present study was obtained from the Ethical Committee of the Jilin Agricultural University,

China. Human kisspeptin-10 amide (amino acid sequence: YNWNSFGLRF-NH_2) was synthesized by Shanghai Qiangyao biotechnology Limited (Shanghai, China).

Experimental design

Experiment IP: Sheep were randomly divided into control (n=4) and feed restriction (FR) groups (n=12). In control group, ewes were fed with standard diet formulated according to China Feeding Standard for sheep (NY/T816-2004) throughout the experiment. Formulation and nutrient compositions of the experimental diet are shown in Table 1. In feed restricted group, ewes were subjected to 40% restriction in daily food intake. Ewes in both groups had free access to water. Thirty days later, 4 ewes from each group were killed by decapitation under pentobarbital anesthesia, and the hypothalamus was immediately dissected out and frozen in liquid nitrogen until processing for RNA analysis. Blood samples (5ml) were also collected and centrifuged at 3000 rpm for 15 min, and serum was separated and then stored at −20℃for later hormone analysis.

Experiment II: The rest ewes under feed restriction were then subjected to pharmacology experiment. One group received intravenous injection of 5 ml of saline (FR+S) (n=4) and the other group was injected with 1 mg of kisspeptin-10 (FR+kp10). Blood samples (5ml) were collected via jugular catheter every 15 min for 180 min (from 30 min before injection to 150 min after injection).

RNA analysis by real-time RT-PCR: Total RNA was isolated from the hypothalamus using a Trizol reagent kit (TAKARA, Dalian, China). cDNA was synthesized with oligo primers at 50ºC using the Super Script III First-Strand Synthesis System (Invirogen Co.). Hypothalamic expression of KiSS-1 and GPR54 mRNAs was determined by fluorescent quantitative PCR. The fluorescent quantitative PCR was performed using the StepOne Real-Time PCR Systems (Applied Bio systems, USA) according to manufacturer's instructions. β-actin was used for reference gene and primers and Taqman probe for β-actin gene were designed according to GenBank (GeneBank accession NO.U39357). Primers and Taqman probes for sheep KiSS-1 and GPR54 genes were designed according to previously published work (Bellingham *et al*. 2009). The information of primers and Taqman probes for β-actin, *KiSS-1* and *GPR54* is elaborated in Table 2. The qPCR reaction conditions were as follows: 95ºC for 10 min, followed by 45 cycles of 30 sec at 95ºC (denaturation step) and 1 min at 60ºC (primer annealing and elongation). Expression of KiSS-1and GPR54 mRNA was quantified by using the comparative CT (cycle threshold) method (Schmittgen and Livak, 2008), and results are presented as the ratio of KiSS-1 or GPR54 to reference gene β-actin mRNA expression.

Hormone measurements: Serum LH levels were determined using sheep LH ELISA Kit (Cusabio biotech CO. Ltd). Serum Lepitn and insulin levels were determined using sheep RIA Kits (Jiuding CO. Ltd). All the processes were performed according to the protocols elaborated in kit manuals.

Statistical analysis: Statistical software SPSS 16.0 (SPSS Inc, Chicago) was used for all statistical analysis. Student's test was performed to determine the significance level of genes expression and hormonal profile between control and treatment groups. The results were expressed as mean ± SD. In general, p values less than 0.05 were considered statistically significant.

RESULTS

Effects of feed restriction on hypothalamic expression of *KiSS-1* and *GPR54* are shown in Figure 1 and Figure 2. Compared to control group, 40% feed restriction significantly ($P<0.01$) decreased hypothalamic expression of *KiSS-1* of ewes [Figure 1(a)]. However, there is no significant difference ($P > 0.05$) on hypothalamic expression of *GPR54* between the two groups [Figure 1(b)]. Feed restriction significantly decreased serum concentration of LH ($P<0.05$), insulin ($P<0.05$) and leptin ($P<0.01$) of ewes, compared to control group (Table 3). Intravenous injection of 1mg of kisspeptin-10 significantly increased the LH secretion of ewes under feed restriction ($P<0.01$) and this LH promotion effect occurred within 15 min of injection, compared to saline injection.

Table 1. Formulation and nutrient compositions of the experimental diet.

Ingredient (%)		Nutrient composition	
Leymuschinensis (%)	40.00	DE[2] (MJ/kg)	11.84
Maize meal (%)	39.43	Crude protein (%)	13.75
Soyabean meal (%)	9.60	Calcium (%)	0.37
wheat bran (%)	4.80	Phosphorus (%)	0.3
corn gluten meal (%)	4.80		
limestone (%)	0.50		
Premix[1] (%)	0.50		
Salt (%)	0.37		
Total (%)	100		

[1] Premix composition: 30 mg Fe/kg; 20 mg Zn/kg; 15 mg Mn/kg; 6 mg Cu/kg; 0.2 mg Co/kg; 0.6 mg I/kg; 9000 IU/kg VA；2000 IU/kg VD；VE 20 IU/kg。

[2] Calculated: DE was calculated based on analyzed values of feedstuffs (NRC, 1994).

Table 2.Probe and primer sequences for qRT-PCR.

Gene	mRNA primer sequence 5´-3´	Product	Reference
KiSS-1	Forward: CTGGTGCAGCGGGAGAAG Reverse: GCGCAGGCCGAAGGA Probe (FAM labeled): ACGTGTCCGCCTACA	57 bp	Bellingham *et al*. 2009
GPR54	Forward: TACATCCAGCAGGTCTCGGTG Reverse: ACGTACCAGCGGTCCACACT Probe (FAM labeled) : CACGTGTGCCACTCTGACCGCC	71 bp	Bellingham *et al*. 2009
β-actin	Forward: TGACGTCGACATCCGCAAAG Reverse: GGAGCCGCCAATCCACAC Probe (FAM labeled) : CCTCTACGCCAACACGGTGCTGTCC	179 bp	U39357; NCBI2009

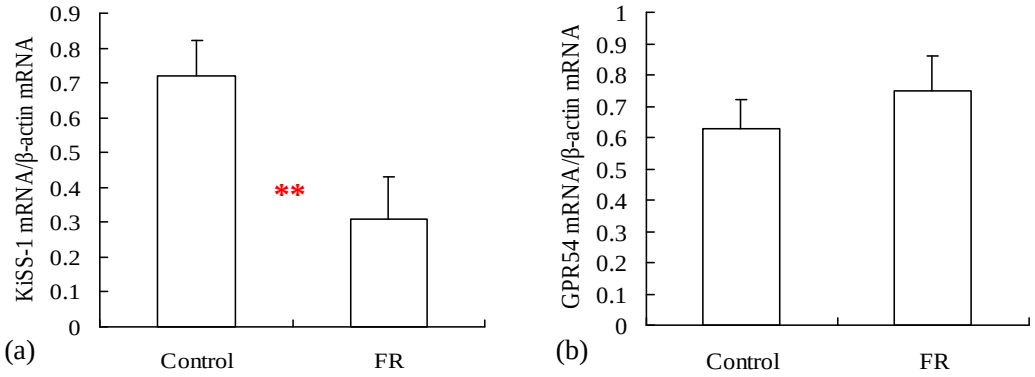

Figure 1.Hypothalamic expression of *KiSS-1*(a) and *GPR54* (b) in prepubertal ewes of control and feed restriction (FR) group.

** P < 0.01 as compared with that in control group.

Table 3.Secretion level of LH, insulin and leptin in ewes.

Group	LH(mIU/ml)	Insulin («IU/ml)	Leptin (ng/ml)
Control	26.23±3.6	20.16±2.96	5.64±0.24
Feed restriction	15.62±4.4*	10.18±2.71*	1.22±0.43**

*P < 0.05, ** P < 0.01 as compared with that in control group.

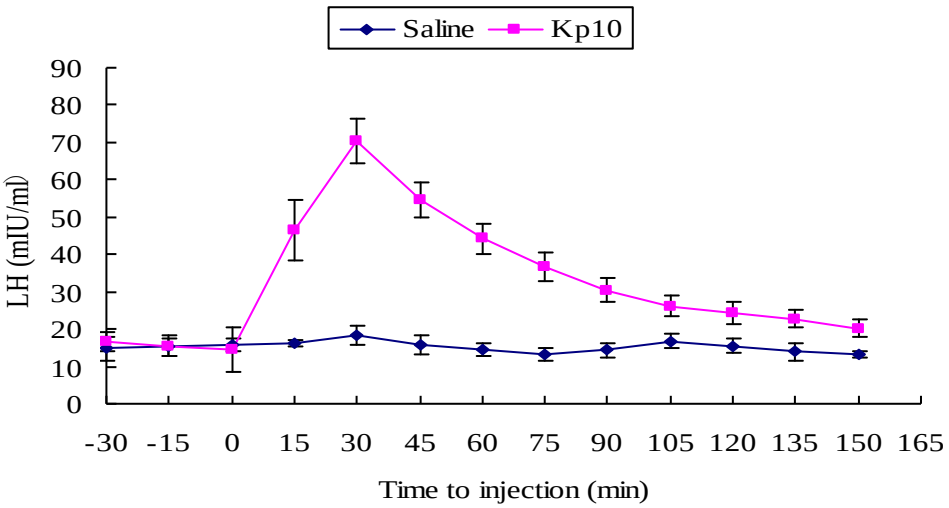

Figure 2. Changes in the serum of LH over 180 min in FR ewes that received an intravenous injection of 5 ml of saline (S) or 1 mg kisspeptin-10 dissolved in saline (Kp10) at 0 min. Concentrations of LH were expressed as mean ± SD.

DISCUSSION

Results of the present study firstly demonstrated that long-term dietary restriction could significantly decreased hypothalamic expression of *KiSS-1* and LH secretion in prepubertal ewes, and injecting with 1mg of kisspeptin-10 could restore the LH secretion.

Data concerning hypothalamic expression of *KiSS-1* and LH secretion is consistent with previous studies in rodents. It has been discovered that malnutrition resulted in a delay in the GnRH and KiSS-1/kisspeptin peaks in juvenile female Sprague-Dawley rats (Lulu *et al*. 2013). Feed restriction (50% of food intake) for 10d, 20d or 30d could also result in decreasing hypothalamic expression of *KiSS-1* and LH release (Ahmed *et al*. 2012). These results indicated that nutrition restriction could suppress hypothalamic expression of *KiSS-1* and LH secretion. Furthermore, unchanged hypothalamic expression ofGPR54 demonstrated in the present study indicates that effect of feed restriction on LH secretion may be due to decreasing hypothalamic expression of *KiSS-1*, not its receptor, *GPR54*.

Our results also showed that feed restriction could decrease level of insulin and leptin in serum of prepubertal ewes, similar to those observed in previous studies (Amstalden *et al*. 2002; Maciel *et al*. 2004; Kiani 2013). It is well known that insulin and leptin play important roles in nutritional metabolism and reproductive performance (Gamba and Pralong, 2006). Studies have shown that insulin and leptin could directly regulate GnRH secretion from hypothalamus and leptin can cancel out the suppressing effect of fasting on LH secretion by augmentation of GnRH (Wójcik-Gładysz *et al*. 2009; Navarro and Kaiser, 2013). However, the exact mechanism of insulin and leptin action on GnRH secretion under nutrition control is still unclear.

The present study alsoshowed that intravenous injectionof 1mg of kisspeptin-10 could restore LH secretion of prepubertal ewes under dietary restriction. The injection dose was based on our previous study (Wang *et al*. 2012). To our knowledge, although administration of kisspeptin has been shown to elicit the secretion of LH in intact adult goats (Redmond *et al*. 2011) and ovariectomized ewes (Arreguin-Arevalo *et al*. 2007), there is no data on prepubertal ewes under feed restriction. Our results are consistent with data from rodents.Interrupting puberty onset by a regimen of undernutrition was able to be restored by repeated administration of kisspeptin to immature female rats (Castellano *et al*. 2005). Similarly, repeated intracerebral administration of kisspeptin-10 to uncontrolled diabetic male rats was capable to restore LH secretion (Castellano *et al*. 2006). These results imply that kisspeptin can restore the low level of LH secretion caused by undernutrition.

Conclusion: It is concluded that the inhibitory effect of LH secretion by long-term feed restriction in prepubertal ewes is probably due to a decrease in hypothalamic expression of *KiSS-1*.

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