Advanced Onset of Puberty in High-Fat Diet-Fed Immature Female Rats - Activation of *KiSS-1* and GnRH Expression in the Hypothalamus -

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ABSTRACT : In mammals, puberty is a dynamic transition process from infertile immature state to fertile adult state. The neuroendocrine aspect of puberty is started with functional activation of hypothalamus-pituitary-gonadal hormone axis. The timing of puberty can be altered by many factors including hormones and/or hormone-like materials, social cues and metabolic signals. For a long time, attainment of a particular body weight or percentage of body fat has been thought as crucial determinant of puberty onset. However, the precise effect of high-fat (HF) diet on the regulation of hypothalamic GnRH neuron during prepubertal period has not been fully elucidated yet. The present study was undertaken to test the effect of a HF diet on the puberty onset and hypothalamic gene expressions in immature female rats. The HF diet (45% energy from fat, HF group) was applied to female rats from weaning to around puberty onset (postnatal days, PND 22-40). Body weight and vaginal opening (VO) were checked daily during the entire feeding period. In the second experiment, all animals were sacrificed on PND 36 to measure the weights of reproductive tissues. Histological studies were performed to assess the effect of HF diet feeding on the structural alterations in the reproductive tissues. To determine the transcriptional changes of reproductive hormone-related genes in hypothalamus, total RNAs were extracted and applied to the semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). Body weights of HF group animals tend to be higher than those of control animals between PND 22 and PND 31, and significant differences were observed PND 32, PND 34, PND 35 and PND 36 ($p<0.05$). Advanced VO was shown in the HF group (PND 32.8±0.37 $p<0.001$) compared to the control (PND 38.25±0.25). The weight of ovaries ($p<0.01$) and uteri ($p<0.05$) from HF group animals significantly increased when compared to those from control animals. Corpora lutea were observed in the ovaries from the HF group animals but not in control ovaries. Similarly, hypertrophy of luminal and glandular uterine epithelia was found only in the HF group animals. In the semi-quantitative RT-PCR studies, the transcriptional activities of *KiSS-1* in HF group animals were significantly higher than those from the control animals ($p<0.001$). Likewise, the mRNA levels of GnRH ($p<0.05$) were significantly elevated in HF group animals. The present study indicated that the feeding HF diet during the post-weaning period activates the upstream modulators of gonadotropin such as GnRH and *KiSS-1* in hypothalamus, resulting early onset of puberty in immature female rats.

Key words : High-Fat(HF) diet, Puberty onset, Female rats, GnRH, *KiSS-1*.

INTRODUCTION

In mammals, puberty is a dynamic transition process from infertile immature state to fertile adult state. The neuroendocrine aspect of puberty is started with functional activation of hypothalamus-pituitary-gonad (H-P-G) hormonal axis, particularly increase in pulsatile release of GnRH from the hypothalamus (Ojeda et al., 1983). After the activation of GnRH pulse generator, the pulsatile gonadotropin secretion from anterior pituitary gland increases, and in turn this increments of gonadotropin induce the normal development and function of gonads. The timing of puberty can be altered by many factors including hormones, endocrine-disrupting chemicals, social cues and metabolic signals (Rasier et al., 2006; Ahmed et al., 2009).

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Since the restriction of food intake significantly delay the puberty onset in many mammalian species including the rat and human, attainment of a particular body weight has been thought as crucial determinant of puberty onset (Frisch & Revelle, 1971).

This decades-old concept has been subsequently modified to more sophisticated form, a critical body fat hypothesis, which proposes that puberty onset is triggered by attainment of a particular percentage of body fat (Frisch et al., 1975; Kirtley & Maher, 1979). The importance of body fat in triggering puberty onset is further reinforced by the finding the indirect role of leptin, a hormone from adipocyte, which allow the pubertal maturation to proceed (Cheung et al., 1997). However, the precise effect of high-fat (HF) diet on the regulation of hypothalamic GnRH neuron during prepubertal period has not been fully elucidated yet.

One strong candidate which mediates the effect of excess dietary fat on the regulation of GnRH neuron is kisspeptin, a peptide product of *KiSS-1* gene. Food deprivation induced a concomitant decrease in hypothalamic *KiSS-1* mRNA levels in prepubertal rats, and chronic treatment with kisspeptin was able to restore vaginal opening (VO), a the first observable sign puberty onset in rodents (Castellano et al., 2005). We hypothesized that continuous feeding of high-fat (HF) diet during post-weaning period might accelerate the puberty onset in female rats via stimulation of hypothalamic kisspeptin and its receptor GPR54 system. We tested this hypothesis by comparing the sexual maturation rate, histology and transcriptional activities of hypothalamic target genes between control and HF-fed animals.

MATERIALS AND METHODS

1. Animals and Treatments

Timely pregnant Sprague-Dawley rats were obtained from Han-Lim Animal (Gyunggi-do, Korea) and reared in our animal facility under conditions of 12-h light/dark cycle(lights on at 07:00 h) and constant temperature of 22±1℃. During pregnancy and lactation, the mothers had free access to normal chow and tap water. At weaning (postnatal day, PND 22) female pups were divided into two groups. The one group was fed with a high fat diet (HF group) which contained 24% fat by weight and provided 45% calories from fat (HFD 45% cal, Han-Lim Animal). The other group (control) was fed a normal chow containing the standard amount of fat (7.0% fat by weight), which provided 16% calories from fat (AIN-93G, Han-Lim Animal). The amount of protein, carbohydrate, fat and vitamins were the same in both diets (Table 1). Body weights were measured daily from weaning to puberty. To determine the day of puberty onset, the animals were daily inspected (PND 30-40) to check vaginal opening (VO). Animals were sacrificed when first estrus stage was observed in HF group (PND 36). After sacrifice, the ovary, uterus, adrenal, kidney, spleen, thymus and liver were

Table 1. Composition of control (AIN-93G) and high-fat diet (HFD 45% cal) used in this study

Formulation	AIN-93G		HFD 45% cal	
	gm %	kcal %	g m%	kcal %
Protein	20	20	24	20
Carbohydrate	64	64	41	35
Fat	7	16	24	45
kcal/kg	4,000		4,776	
Ingredient	g	kcal	g	kcal
Casein (from milk)	200	800	200	800
Corn starch	397.486	1,590	155.036	620
Sucrose	100	400	50	200
Dextrose	132	528	132	528
Cellulose	50	0	50	0
Soybean oil	70	630	25	225
Lard	0	0	175	1,575
Mineral mixture	35	0	35	0
Vitamin mixture	10	40	10	40
TBHQ	0.014	0	0.014	0
L-Cystine	3	12	3	12
Choline bitartrate	2.5	0	2.5	0
Total	1,000	4,000	837.5	4,000

removed and weighed. Hypothalami were immediately removed and placed in solution D (4M guanidine thiocyanate, 25 mM sodium citrate, 0.5% sarkosyl and 0.1 M 2-mercaptoethanol) stored at −70℃ until used for RNA extraction.

2. Histological Studies

Ovaries and uteri were fixed in 4% paraformaldehyde overnight at 4℃ for 24 h. Fixed tissue were dehydrated in ethanol (70%, 80%, 90%, 95%, 100%) and embedded in paraffin block. The tissues blocks were cut at 4-5 ㎛ using microtom (HM350S, MICROM, Germany). Sections were stained with hematoxylin-eosin and observed using a light microscope (BX51, Olympus, JPN).

3. Total RNA Preparation and RT-PCR Analyses

Total RNAs were isolated from hypothalamic samples using the single-step, acid guanidinum thiocyanate-phenol-chloroform extraction method (Chomczynski & Sacchi, 1987). Total RNAs were used in RT-PCR reactions carried out with Maxime™ RT PreMix (Intron, KOR) and Accupower PCR Premix (Bioneer, KOR) according to the manufacturer's instructions. Sequences of the gene primer sets and the annealing temperatures are given in Table 2. As internal control, parallel amplification of GAPDH mRNA was carried out in each sample. PCR-generated cDNA fragments were resolved in 1.5% agarose gels and visualized by ethidium bromide staining. Quantification of the PCR products was performed by densitometric scanning using an image analysis system (Imager Ⅲ-1D main soft ware, Bioneer, KOR), and the values of the specific targets were normalized to those of GAPDH to express arbitrary units (AU) of relative expression.

4. Statistical Analyses

Statistical analysis was performed using Student's *t*-test. Data were expressed as means±S.E., and p value<0.05 denoted the statistically significant difference.

RESULTS

A steady increase in body weight was observed in both control and HF diet-fed animals during entire feeding period (PND 22-PND 36). Between PND 22 and PND 31 body weights of HF diet-fed animals tend to be higher than those of controls without significance, and significant differences were observed PND 32, PND 34, PND 35 and PND 36 (p<0.05, Fig. 1).

In the female rats, VO and ovulation take place when the hormonal axis becomes fully mature, so VO check is convenient method to confirm the puberty onset in female rodents (Halasz et al., 1988). Advanced VO was shown in the HF groups (PND 32.8±0.37, p<0.001) compared to the control group (PND 38.25±0.25, Fig. 2 and Table 3). The

Table 2. Primer sequences used in the semi-quantitative RT-PCR analyses

Gene	Gene Bank No.	Primer sequence	Product size (bp)	AT (℃)
GAPDH	XM_214287	F 5'- CCATCACCATCTTCCAGGAG R 5'- CCTGCTTCACCACCTTCTTG	576	50
KiSS-1	NM_181692	F 5'- ATCTCGCTGGCTTCTTGGCA R 5'- GGAGTTCCAGTTGTAGGCTG	339	62
GPR54	NM_023992	F 5'- TGTGCAAATTCGTCAACTACATCC R 5'- AGCACCGGGGCGGAAACAGCTGC	193	63
GnRH	NM_012767	F 5'- CGCTGTTGTTCTGTTGACTG R 5'- GCTTCCTCTTCAATCAGACG	234	61

F: forward, R: reverse, A.T.: annealing temperature.

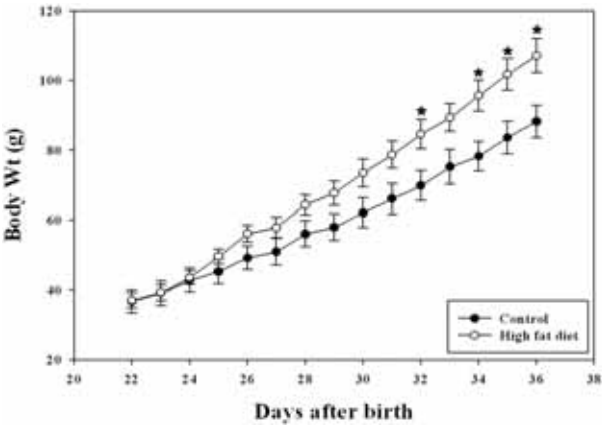

Fig. 1. Changes in body weight of the post-weaning rats fed with control diet or isoaloric HF diet. Values are expressed as mean mean±S.E. (n=4-6 per group). *Significantly different from control group, $p<0.05$.

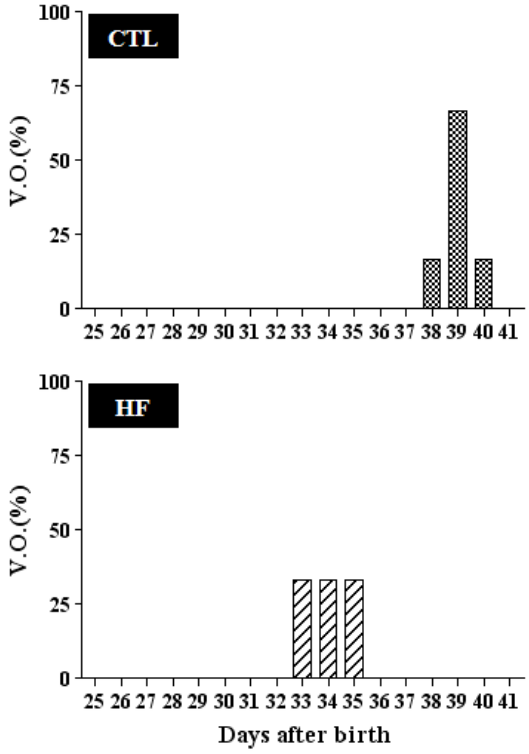

Fig. 2. Dates of vaginal opening in the rats fed with control or HF diet. Bar histograms show the percentage of total individual number per experimental group (n=6).

weights of ovaries (0.27±0.02 ㎎/g BW, $p<0.01$) and uteri (0.36±0.02 ㎎/g BW, $p<0.05$) from HF group animals

Table 3. Body and organ weights of the rats fed with control or HF diet

	CTL	HF
BW (g) at 36-day age	88.25±4.61	107.20±4.92*
Vaginal opening day	38.25±0.25	32.80±0.37***
Tissue weights (㎎/g BW)		
pair of ovaries	0.27±0.02	0.38±0.02**
pair of uterus	0.36±0.02	1.08±0.23*
pair of adrenals	0.25±0.01	0.24±0.02
pair of kidneys	10.67±0.17	9.98±0.23
Spleen	3.18±0.13	3.82±0.26
Thymus	4.03±0.28	4.29±0.19
Liver	40.55±0.92	41.10±2.22

Values were expressed as mean±S.E.(n=4-6).
*Significantly different from control, $p<0.05$.
**Significantly different from control, $p<0.01$.
***Significantly different from control, $p<0.001$.

significantly increased when compared to the weights of ovaries (0.38±0.02 ㎎/g BW) and uteri (1.08±0.23 ㎎/g BW) from control animals, and there was no significant difference in weights of non-reproductive organs between the two groups (Table 3).

Numerous primary, secondary and tertiary follicles were observed in control ovaries (Fig. 3, A & C). Unlike control, several number of corpora lutea was observed in the ovaries from the HF groups (Fig. 3, B & D). This finding indicates the first ovulation had occurred already in HF diet-fed animals while control animals remained in sexually immature state. Well-developed luminal epithelia, increased number of glands and thickened endometrial layers were observed in the uteri from HF group animals compared to those from control animals (Fig. 4. A-D).

In the semi-quantitative RT-PCR studies, the transcriptional activities of *KiSS-1* in hypothalami of HF group were significantly higher than those from the control group ($p<0.001$, Fig. 5, A). Likewise, the mRNA levels of GnRH ($p<0.05$) were significantly elevated in HF group (Fig. 5, C). The mRNA levels of GPR54 in HF group were tended to be higher than control, the difference was not significant (Fig. 5, B).

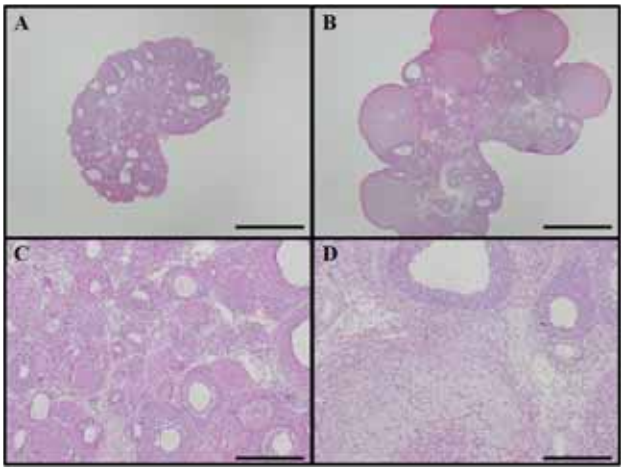

Fig. 3. Microphotographs of ovaries from the control and HF diet-fed rats at PND 36. Stained with hematoxylin and eosin. A & C, ovaries from control rats; B & D, ovaries from HF diet-fed rats. A & B, ×40 magnificaton, bar = 1.0 ㎜; C & D, ×200 magnificaton, bar = 200 ㎛.

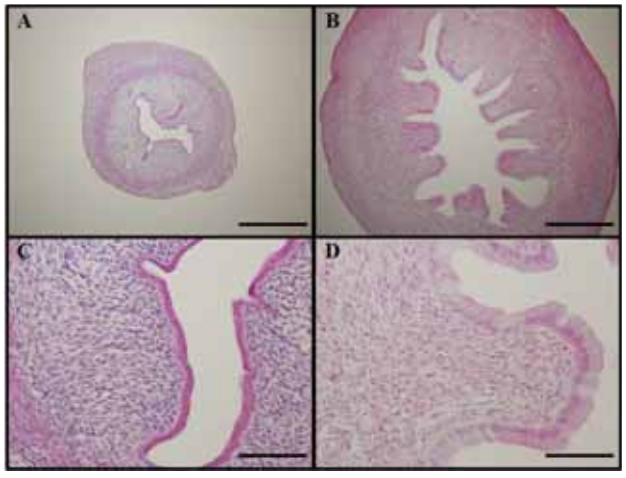

Fig. 4. Microphotographs of uteri from the control and HF diet-fed rats at PND 36. Stained with hematoxylin and eosin. A & C, ovaries from control rats; B & D, ovaries from HF diet-fed rats. A & B, ×100 magnificaton, bar = 400 ㎛; C & D, ×400 magnificaton, bar = 100 ㎛.

DISCUSSION

The hypothalamic GnRH pulse generator is fully operational during perinatal period, and becomes dormant during infancy and childhood. Then, the GnRH pulse generator gradually achieves a frequency compatible with

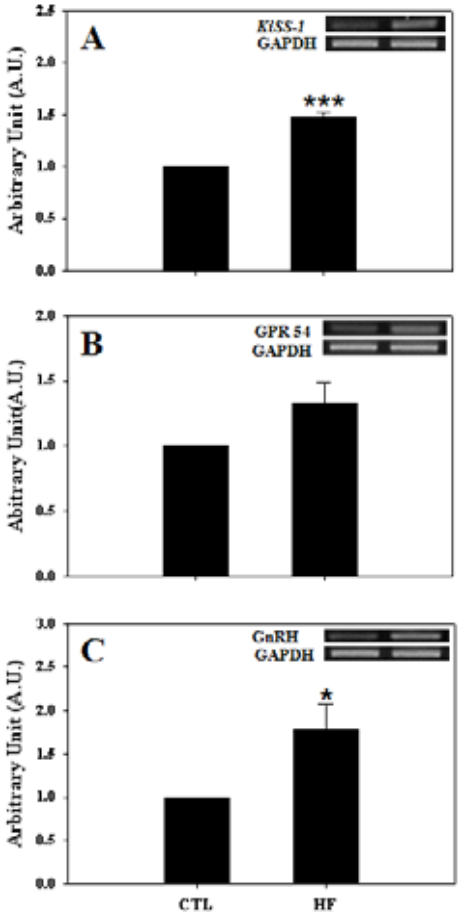

Fig. 5. Effects of dietary HF on the expression of *KiSS-1* (A), GPR54 (B) and GnRH (C) in the hypothalami from the rats sacrificed at PND 36. Semi-quantitative RT-PCR analyses were performed as described in *Materials and Methods*. Values are expressed as mean±S.E. (n=4-6 per group). *Significantly different from control group, $p<0.05$, ***Significantly different from control group, $p<0.001$.

the activation of the pituitary-gonadal axis, and puberty is eventually initiated (Plant, 1994). Though the GnRH pulse generator plays a crucial role in the functional maturation of the ovary, it is the acquisition of ovarian ovulatory capacity which finally determines the timing of puberty onset or the very first preovulatory surge of gonadotropins (Ojeda et al., 1983). In the rat, ovarian development proceeds under the major influence of gonadotropins from anterior pituitary, so rise in circulating estradiol from the maturing ovary leads to VO which is the first observable

consequence accompanying the onset of puberty. The elevated serum estradiol further provokes cell proliferation and differentiation in uterus. In the present study, HF diet-fed rats had VO significantly earlier than did control rats fed isocaloric low fat diet. Our histological studies also confirmed that the follicular maturation in ovaries and uteri with well-developed endometrium and luminal epithelia were evident in HF diet-fed animals. These results indicate that intake of excess dietary fat after post-weaning accelerates the onset of puberty in female SD rats. Regarding these results, previous study using female Osborne-Mendel (O-M) weaning rats fed high fat (HF, 20% corn oil) and low fat (LF, 5% corn oil) diets had reached a similar conclusion (Kirtley & Maher, 1979). However, major difference is exist between the two studies; the SD strain rats used in our study are considered as 'normal' or 'close to normal' at least, while the O-M strain rats are known to be particularly susceptible to dietary-induced obesity (Singer et al., 1997).

In mammals, metabolic factors such as individual's energy balance govern the functional activity of H-P-G reproductive axis. Concerning the timing of puberty, adequate energy stores are a crucial prerequisite for rendering a juvenile animal to proceed successfully through pubertal maturation, as well as to insure continuing reproductive capacity in adulthood (Gamba & Pralong, 2006). In this context, one can speculate that both overweight and lean subjects might experience altered or abnormal puberty onset. However, the precise mechanisms that energy balance mediates the process are not fully understood yet. Majority of information have been provided by using food restriction or fasting animal models, not by excessive food/ energy intake models. We, therefore, hope future studies using HF diet feeding model as in ours will clarify the complex mechanisms of puberty onset.

The pioneering study done by Kennedy & Mitra (1963) demonstrated the relationship existing in a given individual between the timing of puberty and the level of adiposity, and have been further confirmed (Foster et al., 1985; Bronson, 1986). More recent works insisted that leptin, a protein product of *obese (ob)* gene and secreted by adipocytes, plays an important role in the regulation of puberty onset in female rats (Cheung et al., 1997; Almog et al., 2001). The leptin's role in triggering the onset of puberty in female rodents was proven as a permissive rather than direct action on the activation of GnRH neuron (Cheung et al., 2001). Since hypothalamic GnRH neurons do not express leptin receptors (Finn et al., 1998), leptin might act on leptin-sensitive neuron (s) upstream to the GnRH neurons. Neuropeptide Y (NPY) neurons in hypothalamus express functional leptin receptors (Baskin et al., 1999), GnRH neurons express NPY (Y1) receptors (Li et al., 1999), and most importantly, NPY is known to participate in the neuroendocrine network integrating metabolism and reproduction (Gamba & Prolong, 2006).

Another, even more, crucial upstream signal to hypothalamic GnRH neuron is kisspeptin, a product of *KiSS-1* gene. Recent studies clearly show that kisspeptin signalling is an essential regulator of puberty onset as well as preovulatory GnRH neuron activation and the LH surge (Castellano et al., 2005; Clarkson et al., 2008). In rats, most of *KiSS-1* expressing neurons in rostral periventricular area of the third ventricle (RP3V) express ERα mRNA (Adachi et al., 2007). In the present study, we observed that mRNA levels of hypothalamic *KiSS-1* and GnRH were significantly increased in HF diet-fed female rats compared to those of control animals. Our finding indicates that the metabolic signal (s) from dietary fat is correlated with *KiSS-1* and GnRH expression in peripubertal female rats, suggesting another indirect leptin signaling pathway might be exist in the regulation of puberty onset.

Childhood obesity linked early puberty onset is now one of the major public health issues; number of children who suffer from a condition called precocious puberty is growing rapidly. Earlier menarche is known to be associated with increased risk of adult obesity and epigenetic adult-onset diseases such as type 2 diabetes and breast

cancer (Dunger et al., 2005). Puberty initiates the process of epiphyseal closure that terminates linear growth, so children who experience accelerated puberty tend to have short stature and frequently suffer from psychological stress related to concerns about their adult height. A better understanding of the association between metabolic signals from dietary fat and the onset of puberty could not only improve our knowledge on the mechanisms of adolescent reinitiation of GnRH pulse generator activity but also cope with public health issues properly.

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