Effect of High-Fat Diet Feeding on the Reproductive System in Male Rats

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ABSTRACT : It is well known that adipose tissue or body fat has been proved as a crucial component of brain-peripheral axis which can modulate the activities of reproductive hormonal axis in female mammals including rodents and human. Concerning the male reproduction, however, the role of adipose tissue has not been thoroughly studied. The present study was carried out to elucidate the effect of a high-fat (HF) diet on the reproductive system of postpubertal male rats. The HF diet (45% energy from fat, HF group) was applied to male rats from week 8 after birth for 4 weeks. The blood glucose levels, body and tissue weights were measured. Histological studies were performed to assess the structural alterations in the reproductive tissues. To determine the transcriptional changes of reproductive hormone-related genes in hypothalamus and pituitary, total RNAs were extracted and applied to the semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). Body weights ($p<0.01$) and blood glucose levels ($p<0.01$) of HF group were significantly higher than those of control animals. Similarly, the weights of epididymis ($p<0.05$), prostate ($p<0.01$), seminal vesicle ($p<0.01$) in HF group were higher than control levels. The weights of testis were not changed. The weights of kidney ($p<0.001$) and spleen ($p<0.01$) were significantly higher than control levels while the adrenal and pancreas weights were not changed. There were only slight alterations in the microstructures of accessory sex organs; the shape of luminal epithelial cells in epididymis from HF group were relatively thicker and bigger than those from control animals. In the semi-quantitative RT-PCR studies, the mRNA levels of hypothalamic GnRH ($p<0.05$) in HF group were significantly higher than those from the control animals. The mRNA levels of kisspeptin in HF group tend to be higher than control levels, the difference was not significant. Unlike the hypothalamic GnRH expression, the mRNA levels of pituitary LHβ and FSHβ were significantly decreased in HF group ($p<0.05$). The present study indicated that the 4-weeks feeding HF diet during the postpubertal period can alter the hypothalamus-pituitary (H-P) neuroendocrine reproductive system. These results suggest that the increased body fat and the altered leptin input might disturb the H-P reproductive hormonal activities in male rats, and the changed activities seem to be responsible for the changes of tissue weights in accessory sex organs.

Key words : High-fat(HF) diet, Male rats, Postpubertal period, GnRH, Kisspeptin, Gonadotropins, Accessory sex organs

INTRODUCTION

Numerous factors from environment affect the 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). Among them, proper amount of body fat is necessary to trigger the puberty onset in most of female mammals including rodents and human. Based on this phenomenon, a critical body fat hypothesis and its sophisticated versions have emphasized the importance of certain signal (s) which is originated from fat (Frisch et al., 1975; Kirtley & Maher, 1979). After three decades of the initial suggestion, a hormone from adipocytes (i.e., leptin) has been proved as a crucial factor which can modulate the activities of reproductive system consisting of hypothalamic-pituitary-ovary (H-P-O) hormonal axis (Cheung et al., 1997).

Leptin might be a kind of 'translational signal' between metabolism control system and fertility control system (Barash et al., 1996). In female rodents, leptin signaling is known to mediated by several neuropeptide circuits and

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finally modulate GnRH neuronal activity (Quennell et al., 2011). Furthermore, recent evidence indicate that the leptin signaling can alter the kisspeptin expression in the rodent arcuate nucleus (Castellano et al., 2005). In turn, kisspeptin signaling plays an important role in the regulation of GnRH-gonadotropin system (Clarkson et al., 2008). Previous studies including our own demonstrated that high-fat (HF) diet feeding stimulates the reproductive system and sexual maturation in immature female rats (Kirtley & Maher, 1979; Frisch, 1980; Lee et al., 2009). Concerning the male reproduction, however, the role of adipose tissue has not been thoroughly studied.

Several lines of evidence indicate that the presence of strong correlation between obesity and sexual maturation in both boys and girls (Biro et al., 2006; Ahmed et al., 2009). The influence of obesity, however, is sexually dimorphic unless the level of body fat accumulation be immoderate (Wang, 2002). Since the fat accumulation induced by HF diet feeding is positively associated with puberty onset in female rats (Lee et al., 2009), we hypothesized that continuous feeding of high-fat (HF) diet during postpubertal period might be negatively associated with reproductive capacity in male rats by suppressing the GnRH-gonadotropin system. We tested this hypothesis by comparing the expression levels of hypothalamic (kisspeptin and GnRH) and pituitary hormones [common alpha subunit (Cα), LHβ and FSHβ].

MATERIALS AND METHODS

1. Animals and Treatment

Male Sprague-Dawley rats were obtained from Han-Lim Animal (Gyunggi-do, Korea) and acclimated 2 weeks in our animal facility under conditions of 12-h light/dark cycle (lights on at 07:00 h) and constant temperature of 22±1℃. Animal care and experimental procedures were approved by the Institutional Animal care and the use committee at the Sangmyung University in accordance with guidelines established by the Korea Food and Drug Administration.

Eight weeks after birth, male rats 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 (HF diet 45% cal, Han-Lim Animal) for 4 weeks. 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) for 4 weeks. The amount of protein, carbohydrate, fat and vitamins were the same in both diets (Table 1). Body weights were measured daily from the 1st day of feeding. Animals were sacrificed by dacapitation after total 4 weeks of feeding (12 weeks after birth). After sacrifice, the glucose levels in the trunk bloods were measured using the commercial kit (AcuCheck, Roche Diagnostics, Ltd., USA) and the tissues (testis, epididymis, prostate, seminal vesicle, adrenal, kidney, spleen and pancreas) were removed and weighed. Hypothalami and pituitaries

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	1590	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	1575
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

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

2. Histological Studies

Epididymis, prostate and seminal vesicle 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 microtome (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. Total RNAs were used in RT-PCR reactions carried out with Maxime™ RT PreMix (InTron, Korea) and Accupower PCR Premix (Bioneer, Korea) 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 software, Bioneer, Korea), and the values of the specific targets were normalized to those of GAPDH to express arbitrary units (AU) of relative expression.

4. Statistical Analysis

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

Body weights, blood glucose levels and tissue weights are listed in Table 3. Body weights (p<0.01) and blood glucose levels (p<0.001) were significantly higher in HF group animals. Similarly, the weights of epididymis (p<0.05), prostate (p<0.01), seminal vesicle (p<0.01) were significantly higher in HF group animals. The weights of testis and the sperm counts (data not shown) which were squeezed out from epididymis were not changed. Among non-reproductive tissues, the weights of kidney (p<0.001) and spleen (p<0.01)

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

Gene	Gene Bank No.	Primer sequence	Product size (bp)	AT (℃)
GAPDH	NM_017008	F 5'- CCA TCA CCA TCT TCC AGG AG R 5'- CCT GCT TCA CCA CCT TCT TG	557	50
Kisspeptin	NM_181692	F 5'- AAT GGC ACC TGT GGT GAA CC R 5'- GCT GCA CCA GCA CCG ATC CG	236	56
GnRH	NM_012767	F 5'- CGC TGT TGT TCT GTT GAC TG R 5'- GCT TCC TCT TCA ATC AGA CG	234	61
Cg α	BC_063160	F 5'- ATA CTT CTC CAA GCT GGG TG R 5'- CGA CAC TCA GTG CCA TCG CA	294	60
LH-β	NM_012858	F 5'- ATG GAG AGG CTC CAG GGG CT R 5'- CAG AAG AGG AGA AGG CCG GG	425	68
FSH-β	BC168724	F 5'- AAC TGC ACA GGA CAT AGC TG R 5'- ACA GTG GCA TTC AGT GGC TA	344	63

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

Table. 3. Blood glucose levels, body and organ weights of the rats fed with control or HF diet

	CTL	HF
BW (g) at 12 week of age	331.89±5.43	396.11±6.88**
Blood glucose levels	145.63±2.68	162.61±2.36**
Tissue weights (mg/g BW)		
pair of epididymis	0.421±0.005	0.452±0.008*
pair of prostate	0.258±0.013	0.394±0.024**
pair of seminal vesicle	0.159±0.004	0.194±0.005**
pair of testis	1.368±0.012	1.367±0.014
pair of kidney	1.282±0.019	1.380±0.025***
Spleen	0.638±0.027	0.756±0.024**
pair of adrenal	0.021±0.001	0.022±0.000
pancreas	0.650±0.026	0.609±0.029

Values were expressed as mean±S.E.(n=17).
*Significantly different from control, $p<0.05$.
**Significantly different from control, $p<0.01$.
***Significantly different from control, $p<0.001$.

were significantly higher in HF group animals while the weights of adrenal and pancreas were not changed.

There were only slight alterations in the microstructures of epididymis, prostate and seminal vesicles in HF group; the shape of luminal epithelial cells in epididymis from HF group was relatively thicker and bigger than those from control animals (Fig. 1).

In the semi-quantitative RT-PCR studies, transcriptional activities of kisspeptin in hypothalami of HF group tend to be higher than control levels, the difference was not significant (control : HF group= 0.87±0.10AU : 1.66±0.40AU, not significant, Fig. 2A). However, the mRNA levels of GnRH were significantly elevated in HF group (control : HF group= 0.87±0.16AU : 3.59±1.18AU, $p<0.05$, Fig. 2B). The mRNA levels of pituitary common alpha subunit ($C\alpha$) were not changed by HF diet feeding (control : HF group= 0.93±0.17AU : 0.93±0.25AU, not significant, Fig. 3A). However, the mRNA levels of LHβ (control : HF group= 1.18±0.11AU : 0.25±0.10AU, $p<0.001$, Fig. 3B) and FSH β (control : HF group= 0.84±0.23AU : 0.10±0.02AU, $p<0.05$, Fig. 3C) were significantly decreased in HF group animals.

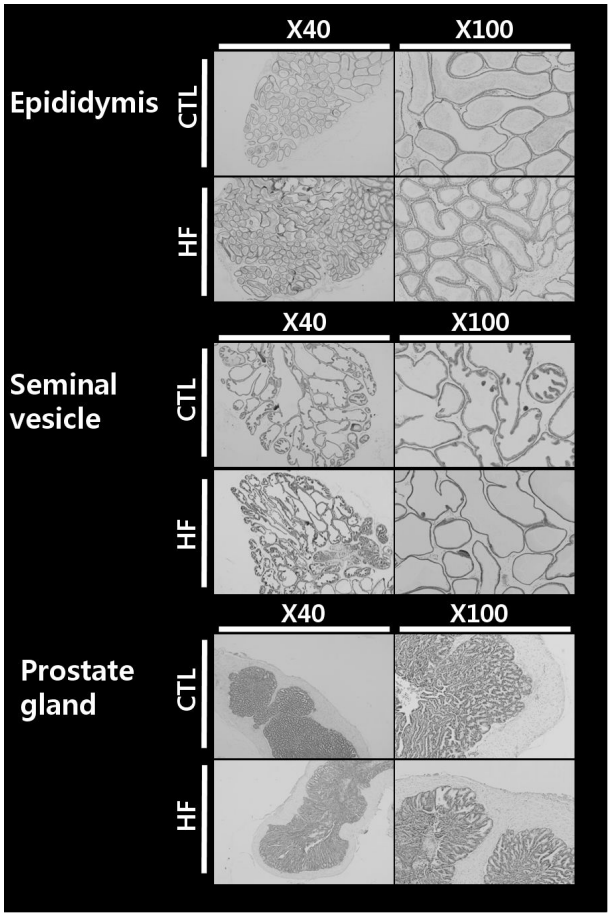

Fig. 1. Effects of dietary high fat feeding on the expressions of kisspeptin (A) and (B) GnRH in the hypothalami from the rats sacrificed at week 12. Semi-quantitative RT-PCR analyses were performed as described in Materials and Methods. CTL, control animals; HF, high-fat fed animals. Values are expressed as mean±S.E. (n=4-6 per group). *Significantly different from control group, $p<0.05$.

DISCUSSIONS

Understanding on the presence of cross-talk between H-P-O reproductive hormone axis and brain-fat metabolic hormone axis was remarkable progress in the endocrine research field over the last two decades. Actually, the possibility of this mutual control was addressed a half century ago. Kennedy & Mitra (1963) demonstrated the relationship existing in a given individual between the timing of puberty and the level of adiposity. After their pioneering work, numerous

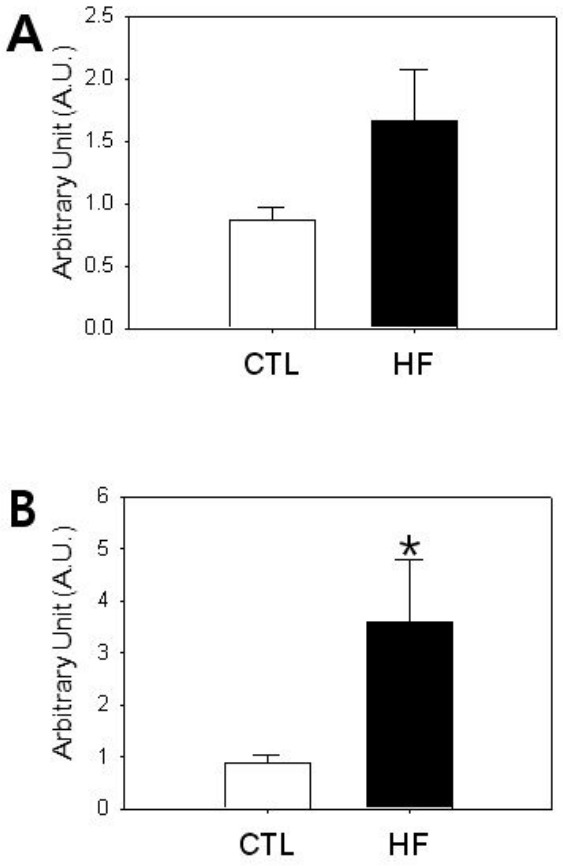

Fig. 2. Effects of dietary high fat feeding on the expressions of pituitary glycoprotein common alpha subunt (Cα, A), LH beta subunit (LHβ, B) and FSH beta subunit (FSHβ, C) in the pituitaries from the rats sacrificed at week 12. Semi-quantitative RT-PCR analyses were performed as described in Materials and Methods. CTL, control animals; HF, high-fat fed animals. 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.

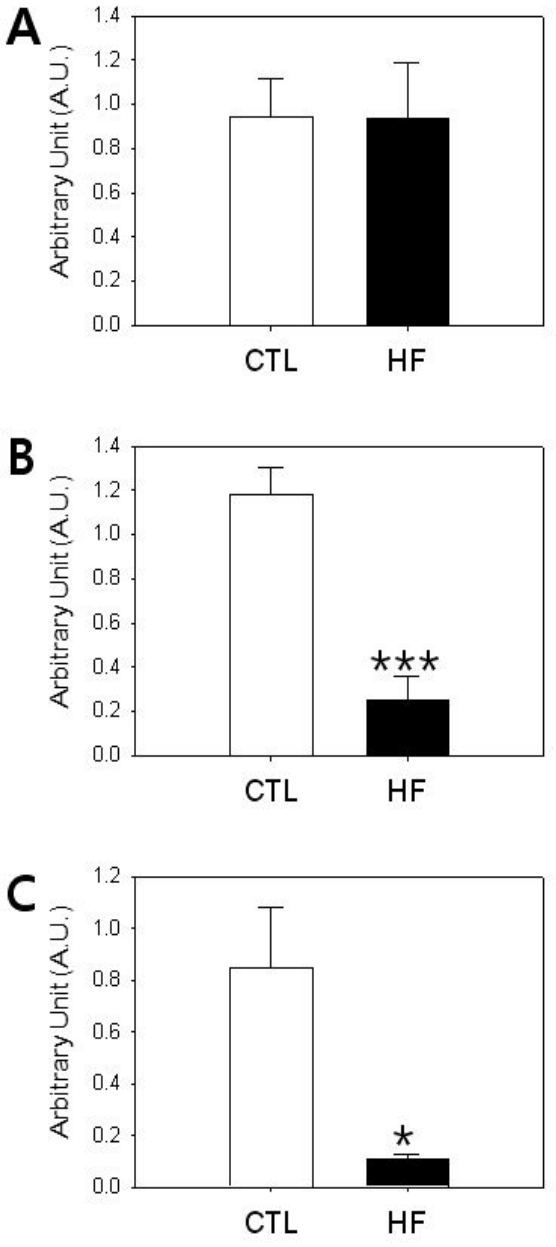

Fig. 3. Microphotographs of epididymis, prostate (ventral part) and seminal vesicle from the control (CON) and HF diet-fed (HF) male rats at week 12. Tissue sections (4-5 ㎛ thick) were prepared by using standard paraffin embedding method. Stained with hematoxylin and eosin.

studies have been performed to elucidate the precise mechanism of the cross-talk in reproductive physiology including puberty onset.

The molecular characterization of the fat-originated signal was finally elucidated by using the *ob/ob* or obese mice at the end of the last century (Cheung et al., 1997). Leptin, a protein product of *obese* (*ob*) gene and secreted by adipocytes, plays a pivotal role in the regulation of energy balance as well as puberty onset in female rats (Almog et al., 2001). Since hypothalamic GnRH neurons do not express leptin receptors (Finn et al., 1998), leptin modulates GnRH

neuronal activity via an indirect action on forebrain neurons such as proopiomelanocortin (POMC), cocaine-and amphetamine-regulated transcript (CART) and most importantly neuropeptide Y (NPY) (Quennell et al., 2011). NPY neurons in hypothalamus express functional leptin receptors (Baskin et al., 1999), and GnRH neurons express NPY (Y1) receptors (Li et al., 1999). So it was plausible that hypothalamic NPY could relay the extrabrain leptin signal and participate in the neuroendocrine network integrating metabolism and reproduction (Gamba & Prolong, 2006).

Kisspeptin, a neuropeptide product of *KiSS-1* gene in brain, is another example of the great achievement in reproductive endocrinology during the last decade. Kisspeptin was originally discovered as a 54-amino acid peptide (kisspeptin-54) or metastin and was found to have metastasis inhibiting activity (Ohtaki et al., 2001). Interestingly, ample of evidence 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). Kisspeptin-54 and its shorter products kisspeptin-10 have been found to potently and directly activate GnRH neurons (Messager et al., 2005). In rodents, on the other hand, food restriction which can alter the level of leptin input significantly decreased hypothalamic kisspeptin mRNA levels (Castellano et al., 2005). Using the double-label *in situ* hybridization for kisspeptin mRNA and the leptin receptor (Ob-Rb) mRNA, Steiner group found that almost one-half (approximately 40%) of kisspeptin mRNA-expressing cells in the arcuate nucleus expressed Ob-Rb mRNA (Smith et al., 2006). So the arcuate kisspeptin neurons might be direct targets for regulation by leptin. In other words, kisspeptin neurons are the very first relay of extrabrain leptin signaling in hypothalamus and NPY neurons are not the case. Although there is some controversy, one can speculate that the leptin signaling is mediated by kisspeptin neurons and the modulated kisspeptin signaling seems to be transferred to NPY and/or POMC neurons and finally to GnRH neurons (Fu & van den Pol, 2010; Kim et al., 2010). Previously, we found that advanced puberty onset with significant elevation of hypothalamic kisspeptin and GnRH expressions is occurred in HF diet-fed immature female rats (Lee et al., 2009). Though the data on leptin levels were unavailable, these findings suggest that the metabolic signal (s) from dietary fat is correlated with hypothalamic kisspeptin and GnRH expressions in peripubertal female rats, supporting the above speculation.

In the present study, we report the significant increase of hypothalamic GnRH and significant decreases of pituitary gonadotropins (LHβ and FSHβ) in HF diet-fed postpubertal male rats. The lowered expressions of pituitary gonadotropins in our study are in good agreement with recently published study which employed HF diet-fed male rat model. The authors demonstrated that long-term (90 and 180 days) HF diet-fed rats exhibited insulin resistance and metabolic syndrome; simultaneously, the animals showed decreased serum LH concentrations, low serum testosterone levels, and elevated serum 17β-estradiol concentrations (Olivares et al., 2010). The data, including our own, from experiments using male rats strongly suggest the presence of sexual dimorphism in the response of reproductive system against the leptin signaling at pituitary level. During the peripubertal period and possibly adolescence, excessive energy accumulation is associated with sexual maturation and HPG axis activity in both male and female rats, but the association differs. There might be positive association in female rats, but a negative one in male rats. Very limited research, however, has been conducted using male rats, so the suggestion should be thoroughly verified by sizable amount of carefully designed tests. In this context, human studies which are dealing obesity-metabolic syndrome and lowered fertility in male will provide certain insights. The mismatch in response to metabolic signal between hypothalamic kisspeptin and pituitary gonadotropins expressions in the present study may imply another sexually dimorphic feature, though we are not allowed to answer at this point.

In conclusion, the present study indicated that the 4-weeks feeding of HF diet during the postpubertal period of male rats can alter the hypothalamus-pituitary (H-P) neuroendocrine

activities in particular reproductive system. These results suggest that the excessive body fat and the altered metabolic input suppress the H-P reproductive system in male rats, and the lowered pituitary gonadotropin expressions seem to be responsible for the changes in tissue weights of accessory sex organs.

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