Effect of Feeding Time Shift on the Reproductive System in Male Rats

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ABSTRACT : Circadian rhythmicity (e.g. secretory pattern of hormones) plays an important role in the control of reproductive function. We hypothesized that the alteration of feeding pattern via meal time shift/restriction might disrupt circadian rhythms in energy balance, and induce changes in reproductive activities. To test this hypothesis, we employed simple animal model that not allowing *ad libitum* feeding but daytime only feeding. The animals of *ad libitum* feeding group (Control) have free access to food for 4 weeks. The day feeding (=reverse feeding, RF) animals (RF group) have restricted access to food during daytime (0900-1800) for 4 weeks. After completing the feeding schedules, body weights, testis and epididymis weights of animals from both group were not significantly different. However, the weights of seminal vesicle (control : RF group = 0.233 ± 0.014 g : 0.188±0.009 g, $p<0.01$) and prostate (control : RF group = 0.358 ± 0.015 g : 0.259 ± 0.015 g, $p<0.001$) were significantly lower in RF group animals. The mRNA levels of pituitary common alpha subunit (Cα; control : RF group = 1.0 ± 0.0699 AU : 0.1923 ± 0.0270 AU, $p<0.001$) and FSHβ (control : RF group = 1.0 ± 0.1489 AU : 0.5237 ± 0.1088 AU, $p<0.05$) were significantly decreased in RF group. The mRNA levels of ACTH were not significantly different. We were unable to find any prominent difference in the microstructures of epididymis, and there were slight alterations in those of seminal vesicles after 4 weeks of reversed feeding when compared to control samples. The present study demonstrates that the shift and/or restriction of feeding time could alter the pituitary gonadotropin expression and the weights of seminal vesicle and prostate in rats. These data suggest the lowered gonadotropin inputs may decrease androgen secretion form testis, and consequently results in poor response of androgen-dependent tissues such as seminal vesicle and prostate.

Key words : Reverse feeding(RF), Male rats, Circadian rhythm, Gonadotropins, Accessory sex organs

INTRODUCTION

Ample of evidence demonstrates that the circadian rhythmicity (e.g. secretory pattern of hormones) plays an important role in the control of reproductive function even in nonseasonal breeders including humans. Circadian rhythms and their clock genes, mostly of hypothalamo-pituitary-gonadal axis, appear to be involved in optimal reproductive performance (Boden & Kennaway, 2006; Urbanski, 2011).

Reproductive function is also gated by the metabolic status in animals; metabolic stress and over-/under-nutrition being frequently coupled to disturbed reproductive maturation (e.g. puberty onset) and/or infertility (Castellano et al., 2009). Several neuroactive peptides (i.e. kisspeptin, NPY/AgRP, POMC/CART and leptin) in hypothalamus seem to participate during communication between brain and reproductive organs/adipocyte, bridging energy homeostasis and reproduction (Hill et al., 2008; Castellano et al., 2010).

Rats and mice are nocturnal rodents, and are widely used in scientific field. These animals consume the majority of their daily food during the dark phase, but light- phase-only feeding (reversed feeding, RF) induces phase inversion of clock and metabolically active genes in peripheral tissues (Damiola et al., 2000; Schibler et al., 2003; Glad et al., 2011).

We hypothesized that the alteration of feeding pattern via meal time shift/restriction might disrupt circadian rhythms in energy balance, and induce changes in reproductive acti-

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vities. To test this hypothesis, we employed simple animal model that not allowing *ad libitum* feeding but daytime only feeding.

MATERIALS & METHODS

1. Animals

Male Sprague-Dawley rats (6 weeks old) were obtained from Hanlim Animal (Gyunggi-do, Korea) and acclimated 2 weeks in our animal facility under conditions of 12-h light/dark cycle (lights on at 0700 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 animals of *ad libitum* feeding group (Control) have free access to food for 4 weeks. The day feeding animals (RF group) have restricted access to food during daytime (0900-1800) for 4 weeks. Food was removed from the feeder at 1800 hour and replaced in the feeder at 0900 hour. All animals were allowed to drink water freely.

When the feeding schedules were over, animals were sacrificed and the tissues (testis, epididymis, prostate, seminal vesicle and kidney) were removed and weighed. Pituitaries were immediately removed and placed in solution D (4 M 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

Epididymi and seminal vesicles 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, Japan).

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 1. As internal control, parallel amplification of GAPDH mRNA was carried out in each sample. PCR-

Table 1. Primer set for semi-quantitative PCR analyses

Gene	Accession number		Nucleotide sequences	Product size (bp)
Cgα	BC_063160	F	ATA CTT CTC CAA GCT GGG TG	294
		R	CGA CAC TCA GTG CCA TCG CA	
LH-β	NM_012858	F	ATG GAG AGG CTC CAG GGG CT	425
		R	CAG AAG AGG AGA AGG CCG GG	
FSH-β	BC_168724	F	AAC TGC ACA GGA CAT AGC TG	344
		R	ACA GTG GCA TTC AGT GGC TA	
ACTH	BC_058443	F	ATG CCG AGA TTC TGC TAC AG	497
		R	AGC TCC CTC TTG AAC TCT AG	
GAPDH	NM_017008	F	CCA TCA CCA TCT TCC AGG AG	557
		R	CCT GCT TCA CCA CCT TCT TG	

F, forward; R, reverse.

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 and tissue weights are listed in Table 2. After 4 weeks of feeding, body weights of animals from both group were not significantly different (control : RF group = 347.10 ± 9.96 g : 333.25±4.904 g). Similarly, the weights of testis (control : RF group = 1.523±0.035 g : 1.539 ± 0.035 g) and epididymis (control : RF group = 0.517 ± 0.048 g : 0.473 ± 0.010 g) were not significantly different. However, the weights of seminal vesicle (control : RF group = 0.233 ± 0.014 g : 0.188 ± 0.009 g, p<0.01) and prostate (control : RF group = 0.358 ± 0.015 g : 0.259 ± 0.015 g, p<0.001) were significantly lower in RF group animals.

Table 2. Body and organ weights of the rats fed *ad libitum* or reverse feeding (day feeding, 0900-1800).

	Control	RF
BW(g) at 12 W of age	347.10±9.96	333.25±4.904
Tissue weights (mg/g BW)		
testis	1.523±0.035	1.539±0.035
epididymis	0.517±0.048	0.473±0.010
seminal vesicles	0.233±0.014	0.188±0.009**
prostate	0.358±0.015	0.259±0.015***
kidneys	1.138±0.050	1.147±0.034

Values were expressed as mean±S.E.(n=7-8).
**Significantly different from control, p<0.01.
***Significantly different from control, p<0.001.

Kidney was chosen as non-reproductive tissue, and the weights were not significantly different (control : RF group = 1.138 ± 0.050 g : 1.147±0.034 g).

In the semi-quantitative RT-PCR studies, the mRNA levels of pituitary common alpha subunit (Cα) were significantly decreased in RF group (control : RF group = 1.0 ± 0.0699 AU : 0.1923±0.0270 AU, p<0.001, Fig. 1A). The transcriptional activities of LHβ in pituitary of RF group tend to be lower than control levels, but the difference was not significant (control : RF group = 1.0 ± 0.3464 AU : 0.4846 ± 0.1391 AU, Fig. 1B). However, the mRNA levels of pituitary FSHβ were significantly lowered by feeding time shift (control : RF group = 1.0 ± 0.1489 AU : 0.5237 ± 0.1088 AU, p<0.05, Fig. 1C). The mRNA levels of ACTH (control : RF group = 1.0 ± 0.1336 AU : 1.0742 ± 0.2672 AU, Fig. 1D) were not significantly different.

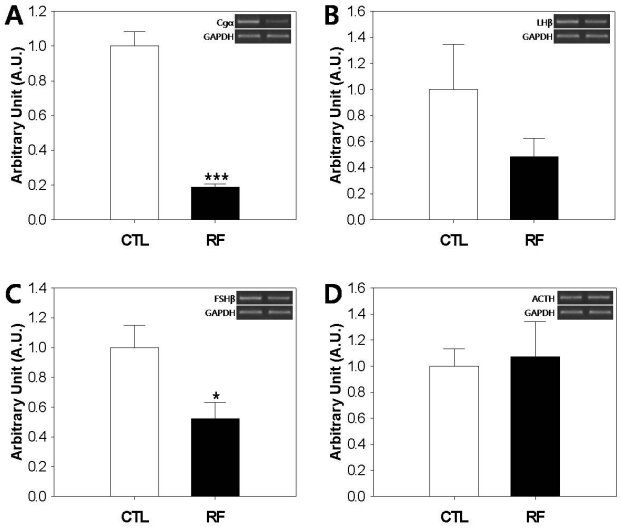

Fig. 1. Effects of reverse feeding on the expressions of pituitary glycoprotein common alpha subunt (Cα, A), LH beta subunit (LHβ, B), FSH beta subunit (FSHβ, C) and ACTH(D) in the pituitaries from the rats sacrificed at week 12. Semi-quantitative RT-PCR analyses were performed as described in Materials and Methods. CTL, control animals; RF, reverse feeding animals. Values are expressed as mean±S.E. (n=6-8 per group). *Significantly different from control group, p<0.05. ***Significantly different from control group, p<0.001.

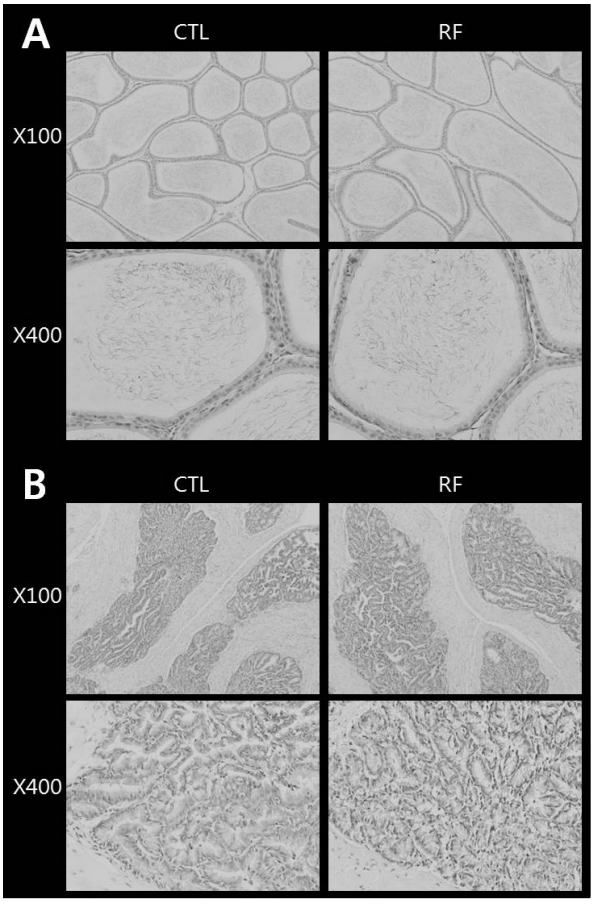

Fig. 2. Microphotographs of epididymis and prostate (ventral part) from the control *ad libitum* feeding animals (CTL) and reverse feeding animals (RF) at week 12. Tissue sections (4-5 ㎛ thick) were prepared by using standard paraffin embedding method. Stained with hematoxylin and eosin.

We were unable to find any prominent difference in the microstructures of epididymis between the control and RF group. There were slight alterations in the microstructures seminal vesicles after 4 weeks of reversed feeding when compared to control samples. The numbers and areas of secretory glands seemed to be increased in seminal vesicles from RF group (Fig. 2).

DISCUSSION

In mammals, homeostatic maintenance is mainly dependent on the circadian rhythmicity in a variety of neuroendocrine and behavioral patterns. For example, hormones and their efficacy are dependent on the temporal secretion pattern, and the regulatory mechanisms underlying the secretion indicate that most body's rhythmic functions are regulated by a coordinated network of central and peripheral circadian pacemakers (Urbanski, 2011). It is now well understood that a light entrainable circadian pacemaker (a classic master clock) is located in the suprachiasmatic nucleus (SCN), receiving retinal inputs and sending neural outputs into pineal gland and peripheral tissues (Boden & Kennaway, 2006). Interestingly, recent studies provide solid evidence on the presence of other circadian system. The induction of this food entrainable oscillator (FEO) by daily feeding schedules does not require the SCN, but FEO do exhibit defining properties of circadian clock control since the SCN lesion could not abolish the properties (Stephan, 2002; Mistlberger, 2011). This food-entrainable oscillator may integrate circadian rhythms, including pituitary hormone (e.g. GH) and adipose hormone (e.g. leptin) secretions, with food availability (Stephan, 2002; Martínez-Merlos et al., 2004; Gooley et al., 2006; Glad et al., 2011). The present study demonstrates that the shift and/or restriction of feeding time could alter the pituitary gonadotropin expression and the weights of seminal vesicle and prostate in rats (Fig. 1A, C and Table 2, respectively). These data suggest the lowered gonadotropin inputs might decrease androgen secretion form testis, and consequently result in poor responses of androgen-dependent tissues such as seminal vesicle and prostate. The pituitary ACTH expression (Fig. 1D) was not changed by RF in the same study suggesting the CRH-ACTH-glucocorticoid system could be under the control of classic light entrainable oscillator, and of less rather than no influence of FEO. Limited evidence suggests that the effectiveness of the RF paradigm is dependent upon the activity of CRH-ACTH-glucocorticoid system (Krieger et al., 1977; Glad et al., 2011).

In the previous study we demonstrated that the 4-weeks

feeding of high fat diet during the postpubertal period of male rats can alter the hypothalamo-pituitary neuroendocrine activities in particular reproductive circuit (Jeon et al., 2011). These results indicate that the excessive body fat and the elevated metabolic input suppress the hypothalamo-pituitary 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. The data also show the amount of food intake (i.e. caloric change) could be a factor controlling the FEO system. Likewise. the present study indicates that the feeding time (i.e. behavioral change) also could be an effective factor. We hypothesize that the LEO and FEO system are not completely independent. These two system may share some components not only at periphery but at somewhat higher levels in brain, or may exert mutual regulation. Indeed, recent study demonstrates the SCN participates actively during food entrainment modulating the response of hypothalamic and corticolimbic structures, resulting in an increased anticipatory response (Angeles-Castellanos et al., 2010). It has been empirically understood that the daily feeding rhythm have the property of being self-reinforcing, because food intake is reciprocally coupled to the circadian clock system; food intake provides cues and elicits physiological responses in certain region (s) of brain, and these can act as entrainment stimuli controlling FEO and possibly LEO, and these higher clocks can control the feeding behaviors and the activities of peripheral organs (Mistlberger, 2011). Further studies on the central clock activities and hypothalamo-pituitary neuroendocrine activities are ongoing using RF model.

Shift and night work generally promotes changes in feeding patterns, resulting in increased food intake during the normal resting phase usually in nighttime (Knutsson, 2003; Vener et al., 1989; Pasqua et al., 2004). Shift and night workers tend to schedule their meals around their working hours, and it is common that their favorite meals are carbohydrate-rich (Lennernas et al., 1995; Karlsson et al., 2001; de Assis et al., 2003). The forced feeding schedules could be a strong entraining influence on peripheral oscillators, on behavior, on visceral rhythms, and on metabolic rhythms, overriding light-dependent rhythmic signals by the SCN-pineal and SCN-peripheral systems, thus it could be a relevant factor promoting internal desynchrony (Damiola et al., 2000; Escobar et al., 1998). In fact, shift or night work is known to associated with hypertension, metabolic syndrome, cancer, and other diseases caused by lowered immune function (Salgado-Delgado et al., 2010). Concerning the night time feeding, night eating syndrome (NES) is more severe disorder in which the affected individuals wake up several times during the night, eat more than 35% of the calories, and are unable to sleep unless they eat something (Milano et al., 2012). The life quality and health of shift/night workers and NES individuals are poor, and the cost to cope with the pathophysiology growing fast. The animal model used in the present study is called 'reverse feeding' rat model, and the RF rats might mimic the shift and night feeding workers' status. Therefore, studies using this model will be helpful to understand the relationship between the food/feeding entrainable rhythm and the occupational diseases.

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