Effect of Reverse Feeding on the Reproductive System in Male Rats

Eun Young Jeon and Sung-Ho Lee†

Department of Green Life Science, Sangmyung University, Seoul 110-743, Korea

ABSTRACT : Circadian timing system plays a major role in a wide range of reproductive function. However it is plausible idea that other environmental and/or internal cue might be simultaneously participated in the optimal regulation of reproductive system. In the present study we extended the reverse feeding (RF) time regimen up to 8 weeks, then measured the general and reproductive indices of the animals. The animals of *ad libitum* feeding group (Control, CON) have free access to food for 4, 6 and 8 weeks, respectively. The day feeding animals (reverse feeding, RF group) have restricted access to food during daytime (09:00-18:00) for 4, 6 and 8 weeks, respectively. When the feeding schedules were over, key indices were measured. After 4 weeks and 8 weeks of feeding, body weights of animals were not significantly different. However, body weights of 6 weeks RF animals were significantly smaller than those of control animals (CON : RF = 333.46±12.71 g : 289.91±8.31 g, $p<0.01$). The blood glucose levels of 4 weeks RF animals were significantly decreased compared to the levels of control animals (CON : RF = 161.4±2.7 mg/dL : 176.7±5 mg/dL, $p<0.01$) while the levels of 6 weeks RF and 8 weeks RF animals were not different form those of control animals. Reproductive and non-reproductive tissue weights from 6 weeks RF group were significantly lowered than those from CON group (testis, CON : RF = 1.4714±0.0174 g : 1.3724±0.0168 g, $p<0.001$; epididymis, CON : RF = 0.3574±0.0059 g : 0.3243±0.0068 g, $p<0.001$; seminal vesicle, CON : RF = 0.1655±0.0068 g : 0.1328±0.0054 g, $p<0.001$; prostate, CON : RF = 0.3350±0.0231 g : 0.2528±0.0143 g, $p<0.01$). After 4 weeks and 8 weeks of reverse feeding, sperm counts in RF animals were markedly reduced than those in control animals[CON 4W : RF 4W = 121.17±9.96 ($\times 10^6$) : 50.86±9 ($\times 10^6$), $p<0.001$; CON 8W : RF 8W= 138.69±9.8 ($\times 10^6$) : 108.94±4.22 ($\times 10^6$), $p<0.001$]. Present study indicates that RF may induce an adaptable metabolic stress and cause impairment of androgen-dependent reproductive tissues. On-going longitudinal studies will allow a better understanding of the how does mealtime shift affect the reproductive function and exact nature of adaptation.

Key words : Reverse feeding (RF), Male rats, Tissue weight, Blood glucose level, Sperm counts

INTRODUCTION

Circadian timing system plays a major role in a wide range of reproductive function. Circadian rhythms and clock genes appear to be involved in optimal reproductive performance, but there are sufficient redundancies in their function that many of the knockout mice produced do not show overt reproductive failure (Boden & Kennaway, 2006). So it is plausible idea that other environmental and/or internal cue might be simultaneously participated in the regulation of reproductive system.

Growing body of evidence support the idea that reproductive function is also tightly correlated with the metabolic status in animals. Metabolic stress and over-/ under-nutrition are frequently coupled to disturbed reproductive maturation such as puberty onset and even infertility (Castellano et al., 2009). Severe food restriction (FR, ≥ 50%), for example, decreased serum testosterone and LH levels resulting negative effects on androgen-dependent male reproductive organs (Grewell et al., 1971; Howland, 1975; Glass et al., 1986; Levin et al., 1993; Santos et al., 2004). In fact, normal function of reproductive (hypothalamus-pituitary-gonadal) axis largely depend on the normal energy balance, which presupposes sufficient food intake, reasonable energy con-

† Corresponding author: Sung-Ho Lee, Department of Green Life Science, Sangmyung University, 7 Hongji-dong, Jongrho-gu, Seoul 110-743, Korea. Tel: +82-2-2287-5139, Fax: +82-2-2287-0098, E-mail: shlee@smu.ac.kr

sumption and average thermoregulatory costs (Michalakis et al., 2012).

Previously we demonstrated that the shift and/or restriction of feeding time during relatively short-erm period (4 weeks) could alter the pituitary gonadotropin expression and the weights of seminal vesicle and prostate in rats (Kwak & Lee, 2012). We hypothesized that this reverse feeding (RF) regimen could induce adaptable behavioral patterns as shown in shift workers although it is quite problematic (Antunes et al., 2010). Consequently, one can assume that the initial homeostatic status of the animal should be somewhat altered through necessity. In the present study we extended the reverse feeding time regimen up to 8 weeks, then measured the general and reproductive indices of the animals.

MATERIALS AND METHODS

1. Animals

Male Sprague-Dawley rats (3 weeks old) were obtained from Hanlim Animal (Gyunggi-do, Korea) and acclimated 1 week 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.

Four weeks after birth, male rats were divided into two groups. The animals of *ad libitum* feeding group (Control, CON) have free access to food for 4 (CON 4W), 6 (CON 6W) and 8 weeks (CON 8W), respectively. The day feeding animals (reverse feeding, RF group) have restricted access to food during daytime (09:00-1800) for 4 (RF 4W), 6 (RF 6W) and 8 weeks (RF 8W), respectively. Food was removed from the feeder at 1,800 hour and replaced in the feeder at 0900 hour. All animals were allowed to drink freely. When the feeding schedules were over, animals were sacrificed and the tissues (testis, epididymis, prostate, seminal vesicle, kidney, adrenal and spleen) were removed and weighed.

2. Measurement of Blood Glucose Levels

The trunk blood samples were collected and centrifuged at 3,000 × g for 15 min. The measurement of blood glucose concentrations was carried out according to the commercial instructions for the ACCU-CHEK Compact Plus analyzer (Germany).

3. Epididymal Sperm Count

The caudal epididymal sperm suspension is prepared in 1 ml of phosphate buffered saline (PBS) at pH 7.2. The sperm count was determined in a hemocytometer. The suspension (1 ml) was diluted 1:100 with PBS, and was charged into a counting chamber. The total sperm count in eight squares of 1 mm^2 each was determined and multiplied by 5×10^4 to get the total count.

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

Significant pathophysiological change was not noted in both group throughout the study period. Changes in body weights are shown in Fig. 1. After 4 weeks and 8 weeks of feeding, body weights of animals from both group were not significantly different (CON 4W : RF 4W = 242.55± 5.73 g : 238.1±3.74 g; CON 8W : RF 8W = 339.89±7.69 g : 330.61±4.62 g, respectively). However, after 6 weeks body weights of RF animals were significantly smaller than those of control animals (CON : RF = 333.46±12.71 g : 289.91±8.31 g, $p<0.01$).

The blood glucose levels of RF 4W animals were significantly decreased compared to the levels of control animals (CON : RF = 161.4±2.7 mg/dL : 176.7±5 mg/dL, $p<0.01$, Fig. 2) while the levels of RF 6W and RF 8W

animals were not different form those of control animals (CON 6W : RF 6W = 151.8±8.5 mg/dL : 159.8±1.9 mg/dL; CON 8W : RF 8W = 153.0±4.9 mg/dL : 159.1±5.4 mg/dL).

Reproductive and non-reproductive tissue weights are listed in Table 1. There was no significant difference between the CON 4W group tissues and RF 4W group tissues. In contrast, reproductive and non-reproductive tissue weights from 6 weeks RF group were significantly lowered than those from CON group (testis, CON : RF = 1.4714± 0.0174 g : 1.3724±0.0168 g, $p<0.001$; epididymis, CON : RF = 0.3574±0.0059 g : 0.3243±0.0068 g, $p<0.001$; seminal vesicle, CON : RF = 0.1655±0.0068 g : 0.1328±0.0054 g, $p<0.001$; prostate, CON : RF = 0.3350±0.0231 g : 0.2528± 0.0143 g $p<0.01$). In 8 weeks feeding animals, only seminal vesicle and prostate weights from RF group were significantly lowered that those from CON group (seminal vesicle, CON : RF = 0.2401±0.0139 g : 0.1836±0.0091 g, $p<0.01$; prostate, CON : RF = 0.3505±0.0146 g : 0.2517± 0.0146 g, $p<0.001$). The weights of other tissues were not significantly different.

After 4 weeks of feeding, sperm counts in RF animals were markedly reduced than those in control animals [CON : RF = 121.17±9.96 ($\times10^6$) : 50.86±9 ($\times10^6$), $p<0.001$, Fig. 3]. In contrast, 6 weeks feeding did not affect the sperm count in both group [CON : RF = 94.67±5.8 ($\times10^6$) : 84.67±2.05 ($\times10^6$)]. However, 8 weeks feeding resulted in significant decrease in sperm counts of RF group animals [CON : RF = 138.69±9.8 ($\times10^6$) : 108.94±4.22 ($\times10^6$), $p<0.001$].

DISCUSSION

In the present study, 4 weeks and 8 weeks of RF failed to induce changes in body weight but 6 weeks of RF

Table 1. Reproductive and non-reproductive tissue weights of the rats fed *ad libitum* (CON, control) or reverse feeding (RF; day feeding, 0900-1800)

Week	4W		6W		8W	
Group (n=12)	CON	RF	CON	RF	CON	RF
Reproductive organ						
Testis (g)	1.2256 (±0.0203)	1.2315 (±0.0374)	1.4714 (±0.0174)	1.3724*** (±0.0168)	1.5427 (±0.0361)	1.5149 (±0.0348)
Epididymis (g)	0.2438 (±0.0452)	0.1927 (±0.0065)	0.3574 (±0.0059)	0.3243*** (±0.0068)	0.4705 (±0.0081)	0.4686 (±0.0103)
Seminal vesicle (g)	0.0921 (±0.0044)	0.0816 (±0.0052)	0.1655 (±0.0068)	0.1328*** (±0.0054)	0.2401 (±0.0139)	0.1836** (±0.0091)
Prostate (g)	0.1512 (±0.0105)	0.1495 (±0.0131)	0.3350 (±0.0231)	0.2528** (±0.0143)	0.3505 (±0.0146)	0.2517*** (±0.0146)
Non-reproductive organ						
Kidney (g)	1.0105 (±0.0472)	0.9219 (±0.0145)	1.2246 (±0.0335)	1.0412*** (±0.0187)	1.0873 (±0.0402)	1.1420 (±0.0377)
Adrenal (g)	0.0168 (±0.0006)	0.0161 (±0.0006)	0.0222 (±0.0006)	0.0184*** (±0.0004)	0.0170 (±0.0010)	0.0236 (±0.0055)
Spleen (g)	0.6169 (±0.0401)	0.5323 (±0.0267)	0.7187 (±0.0515)	0.5165** (±0.0545)	0.5897 (±0.0374)	0.6175 (±0.0209)

Values were expressed as mean±S.E. (n=12).

**, Significantly different from control, $p<0.01$.

***, Significantly different from control, $p<0.001$.

induced signifiant lowered gain of body weight (Fig. 1). This result indicate that the animals have developed adaptation to the RF regimen at 8 weeks after experiencing the alteration of energy metabolism around 4 weeks. Since the blood glucose level was significantly higher only in 4 weeks RF animals, hyperglycemia precedes and seems to cause the lowered body weights in 6 weeks RF animals. *In vivo* human experiments show that insulin secretion oscillates to match anticipated activity level for a normal day, showing greatest secretion during the light-hours (Peschke & Peschke, 1998). Their phase-response studies show phase-shifts of circadian insulin secretion with approximately 9 h phase advance, and thereafter the circadian period was maintained while the amplitude was enhanced. More recently, the authors demonstrated that a robust circadian expression of clock genes (e.g. Per1 and Bmal1) and the probable existence of a peripheral oscillator in the pancreas (Mühlbauer et al., 2004).

Our study show that the 6 weeks RF induced significant weight losses of both reproductive and non-reproductive tissues (Table 1). These effects did not persist for 8 weeks RF in non-reproductive tissues (kidney, adrenal and spleen) while some reproductive tissues (seminal vesicle and prostate) were still notably smaller than control tissues. Prostate and especially seminal vesicle are known to be highly androgen-sensitive tissues because of their large proportion of glandular luminal contents (Rehm et al., 2008). In this context, one can postulate that the decreased synthesis/action of androgen in RF animals. In similar experimental regimen such as FR (≥ 50%), the metabolic stress decreased serum testosterone resulting negative effects on androgen-sensitive male reproductive organs (Grewell et al., 1971; Santos et al., 2004). Concerning the serum gonadotropin level, some studies revealed decreased LH in FR animals while others shown no changes using the same regimen (Howland, 1975; Glass et al., 1986; Rehm et al., 2008). Previously we demonstrated that RF could result in significant decrease of mRNAs for pituitary common alpha subunit and FSH-beta subunit, but failed to alter the mRNA levels of LH beta and ACTH (Kwak & Lee, 2012).

Epididymal sperm counts in RF animals markedly reduced after 4 weeks of feeding, then 2 weeks later, the numbers rose to the normal range, and finally lowered significantly than those of control animals after total 8 weeks of feeding (Fig. 3). Unlike the most of physiological indices, the mature sperm production take relatively long time. In case of rat, whole spermatogenic cycle is of 50 days (Clermont & Harvey, 1965). Definitely, sperm production is androgen-dependent process, so reductions of sperm counts in 4 weeks and 8 weeks RF animals are consequential. The normal level of sperm counting in 6 weeks RF animals may reflect the adaptation of animals to the metabolic stress though it is unable to guarantee full recovery.

Metabolic syndrome (MS), also known as metabolic syndrome X, is a combination of medical disorders that increase the risk of developing cardiovascular disease and diabetes. It is now well accepted that the MS prevalence in the USA to be an estimated 25% of the population and prevalence increases with age (Ford et al., 2002). MS can also influence male reproductive system profoundly. Numerous studies have revealed that serum testosterone levels are

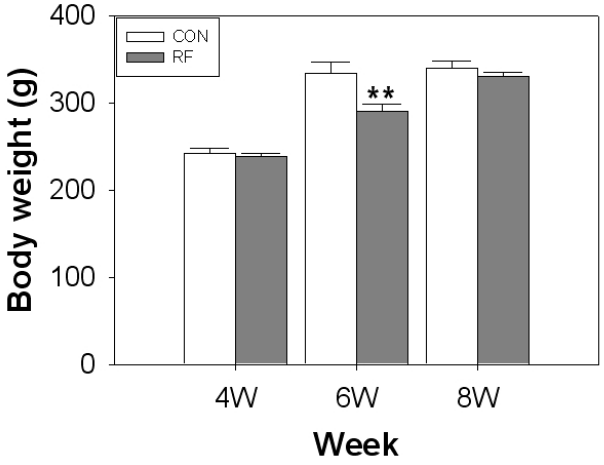

Fig. 1. Body weights of *ad libitum* feeding (CON, control) and reverse feeding (RF) male rats by cohort (mean±S.E., n=12). Cohorts were defined by the feeding period 4, 6 and 8 weeks, respectively. **, Significantly different from control group, $p<0.01$.

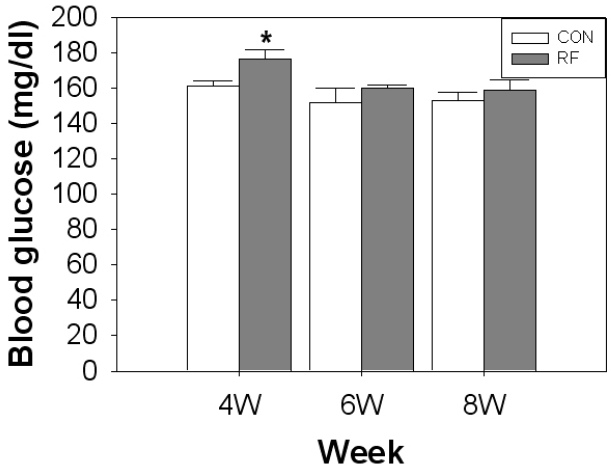

Fig. 2. Effects of reverse feeding on changes in blood glucose levels. The trunk blood samples were collected and centrifuged. The measurement of plasma glucose concentrations was carried out by using commercial kit. CON, control animals; RF, reverse feeding animals. Values are expressed as mean±S.E. (n=12 per group). *, Significantly different from control group, $p<0.05$.

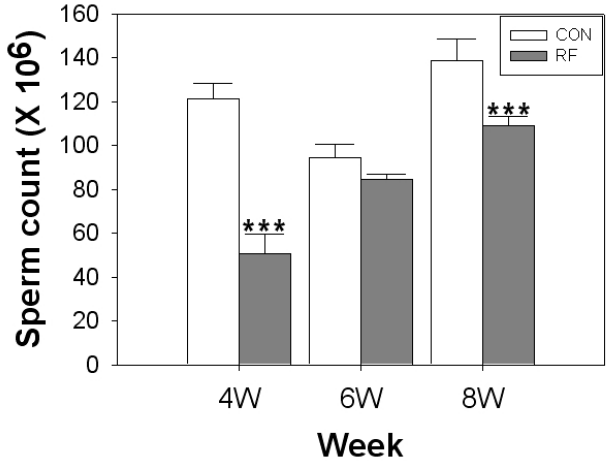

Fig. 3. Evaluation of sperm counts. The caudal epididymal sperm suspension was diluted in PBS (pH 7.2), and the sperm count was determined in a hemocytometer. CON, control animals; RF, reverse feeding animals. Values are expressed as mean±S.E. (n=12 per group). ***, Significantly different from control group, $p<0.001$.

significantly decreased in obese men (for review: Teerds et al., 2011; Michalakis et al., 2012), probably more androgen is converted to estrogen via aromatization in pheripheral fat (Bray, 1997). On the other hand, as shown in FR animal studies, undernutrition also resulted in low testosterone production and action. Therefore, over- and under-nutrition seem to be common metabolic stress which can affect male reproductive function, even causing infertility. RF regimen allows the shift and/or restriction of feeding time, and could drag down the pituitary gonadotropin expression and the weights of seminal vesicle and prostate in rats (Kwak & Lee, 2012). Recent evidence clearly show that the feeding cues alter clock gene oscillatios, and the entrainment to feeding is suprachiasmatic nucleus (SCN)-independent (Escobar et al., 1998; Damiola et al., 2000; Gooley et al., 2006). In this regard, one can hypothesize that FR gradually alters the SCN-independent, peripheral food-entrainable clock system, following the modification of the down-stream factors activities which are responsible for the maintenance of optimal reproductive function (e.g. pituitary gonadotropins and testicular steroidogenesis controlling factors) under the influence of new clocks-setting.

Shift work results in not only a sleep deprivation but also mealtime reschedule. Shift workers tend to eat their meals around their working hours, and show preference for carbohydrate-rich foods (Lennernas et al., 1995; Karlsson et al., 2001; de Assis et al., 2004). Concerning the negative health effects of shift works, most attention has been focused on the risk of chronic disease, including cancer, cardiovascular disease, MS and diabetes (Wang et al., 2011). Growing interest, however, on the relationship between reproduction and shift work is evitable (Rüdiger, 2004; El-Helaly et al., 2010; Lawson et al., 2011). We expect the shift work problems will come under life style factors as a major health threatening issue in the near future. In conclusion, present study indicates that RF may induce an adaptable metabolic stress and cause impairment of androgen-dependent reproductive tissues. On-going longitudinal studies using animal model with subdivided time points will allow a better understanding of the how does mealtime shift affect the reproductive function and exact

nature of adaptation such as rebounding of tissue weights and serum hormone levels.

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