Reproductive aging of hypothalamus-pituitary-testicular axis in middle-aged male rats

Patteera Wititsuwankul[1], Suchinda Malaivijitnond[2], Sukanya Jaroenporn[3] and Taratorn Fainanta[4]

[1]Master of Science Program in Zoology, Department of Biology, Faculty of Science, Chulalongkorn University, manow1988@gmail.com

[2]Department of Biology, Faculty of Science, Chulalongkorn University, suchinda.m@chula.ac.th

[3]Department of Biology, Faculty of Science, Chulalongkorn University, sjaroenporn@yahoo.com

[4]Biological Sciences Program, Faculty of Science, Chulalongkorn University, taratorn.jeng@gmail.com

---

ABSTRACT

Androgen deficiency, an indicator of reproductive aging in males, is not particularly detectable, though serum testosterone levels were gradually and progressively decreased with advancing age. The major organ of androgen production is testis which is regulated via hypothalamic-pituitary-testicular (HPT) axis. Thus, this study was to search when the reproductive aging can be detected in male rats during the transition into the middle age in association with alterations of pituitary luteinizing hormone (LH) production and transcript expression of reproductive hormone-related genes in hypothalamus. Male Sprague-Dawley rats at 4 (young age), 6, 8, 10 and 12 (middle age) months old were subjected for this study. In each age of rats, blood was collected for serum testosterone and LH assays, mRNA levels of gonadotropin releasing hormone (*Gnrh1*) gene at preoptic area (PoA), and kisspeptin (*Kiss1*) and androgen receptor (*Ar*) genes at anteroventral periventricular nucleus (AVPV) and arcuate nucleus (ARC) of hypothalamus were measured. Rats showed a significant reduction of serum testosterone level at 12 months old, though the levels were marginally decreased from 8 months old. Serum LH and PoA-*Gnrh1* mRNA levels were significant declined from 8 months old. AVPV-*Kiss1* mRNA level was significantly decreased at 12 months old. *Ar* mRNA levels in AVPV and ARC were significantly decreased from 8 and 10 months old, respectively. Our study depicts that reproductive aging can be first detected at middle age of male rats, and mechanisms of occurrence is initiated earlier (at 8 months old) by the deterioration at the hypothalamus and pituitary levels.

Keywords: Androgen deficiency, hypothalamus, kisspeptin, LH, testosterone

1. Introduction

Reproductive aging is a cessation of reproduction which can be seen at a menopausal period in females when the ovarian function and serum estrogen levels were abruptly declined (Kermath and Gore, 2012). Although the testicular function and serum androgen levels were gradually and progressively decreased with advancing age, the reproductive aging was not particularly detectable in males (Harman et al., 2001; Morley and Perry, 2000), and

some claimed that andropause did not exist in men (Vermeulen and Kaufman, 1995). Many age-related diseases such as osteoporosis, Alzheimer's disease and coronary atherosclerosis were attributed to the reproductive aging, specifically androgen deficiency, in men (Lv et al., 2016; Rosano et al., 2006). Mechanism of progression of the diseases and the therapeutic agents were extensively explored (Cherrier et al., 2001). Thus, if the initiation of reproductive aging in men can be found, the diseases should be prevented sooner.

Androgens are mainly synthesized in the testis which is regulated by the hypothalamic-pituitary-testicular (HPT) axis (Vermeulen and Kaufman, 1995). The major androgen in males is testosterone. The production of androgens is started by that *Gnrh1*-expressing neurons in the preoptic area (PoA) of the hypothalamus produce gonadotropin releasing hormone (GnRH) and secrete into the median eminence. The secretion of GnRH is regulated by kisspeptin, which encodes by *Kiss1* gene at the anteroventral periventricular nucleus (AVPV) and arcuate nucleus (ARC) regions of the hypothalamus (Oakley, Clifton and Steiner, 2009). GnRH releases in the pulsatile pattern to stimulate the synthesis and secretion of luteinizing hormone (LH) in the anterior pituitary gland (Seeburg et al., 1987; Vale, Rivier and Brown, 1977). LH is secreted and transported to testis via blood circulation and binds with LH receptors on Leydig cells in the testis to stimulate testosterone production (Jin and Yang, 2014). To this end, testosterone has a negative feedback effect on hypothalamus and pituitary gland to inhibit GnRH and gonadotropin expression via androgen receptor (AR) (Tilbrook and Clarke, 2001; Veldhuis, Urban and Dufau, 1992). Recent studies denoted that *Kiss1*- and *Gnrh1*-expressing neurons were a direct target of sex steroid hormones because of the co-expression of androgen receptor (*Ar*) gene (Poletti et al., 2001; Smith et al., 2005).

Although many researchers assessed a male reproductive aging at each level of the HPT axis and did comparison between young, middle-aged and aged males (Munemoto et al., 2015), there is virtually no information on which age of animals the reproductive aging was detected, and changes of the whole complex of the HPT axis.

2. Objectives of the study

This study was to search when the reproductive aging occurred in male rats during a transition into the middle age and the initiative association with the HPT axis.

3. Materials and methods

Animals

Adult male Sprague-Dawley rats, 2 months old, were purchased from the National Laboratory Animal Center, Mahidol University, Thailand. Rats were reared in individually ventilated cage (IVC) systems in a room with controlled lighting (12:12 hour light-dark cycle) and temperature (22±1°C) at Chulalongkorn University Laboratory Animal Center (CULAC), Thailand. The rat were fed with a standard rodent diet and water *ad libitum*. They were reared until they became 4, 6, 8, 10 and 12 months old and euthanized. The whole brains were immediately collected and kept frozen at -80 °C until the hypothalami were isolated and determined mRNA

expression levels. Blood samples were collected for serum LH and testosterone level assays. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the CULAC (Protocol number: 1573004).

Hypothalamus collection and isolation

The transverse section of the brain was performed using stainless steel brain matrices with 200-µm thickness. The section was started from bregma +5.64 mm to bregma -6.60 mm. Each section was dissected the region of hypothalamus including PoA, AVPV and ARC according to the rat brain Paxinos Atlas (Paxinos and Watson, 2005) and placed in a frozen Eppendorf tube. Each brain region was extracted for a total RNA using 1,000 µl TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The quantity and the purity of RNA samples were checked by spectrometer based on optical density (OD) measurements at 260 and 280 nm wavelength. The 260/280 ratio of 2.0±0.1 was accepted for RNA purity.

Transcript expression levels were examined using two-stage qrtRT-PCR. In the first stage, 400 ng of each region of hypothalamus total RNA sample was reverse transcribed to cDNA in a 20 µl reaction mixture containing 4 µl of 5xRT Buffer, 1 µl of dNTP mix, 1 µl of Random Hexamer Primer Mix, 1 µl of Oligo (dT) 18 Primer Mix, 1 µl of reverse transcriptase (200 U/µl) and 1 µl of RNase inhibitor (10 U/µl), using the Tetro cDNA Synthesis kit (Bioline Reagent Ltd., UK). The samples were incubated for 10 min at 25 °C, 30 min at 45 °C, and finally 5 min at 85 °C. The obtained cDNA was diluted in five volumes of DEPC-treated water prior to use.

The second stage was performed using a StepOneTM Plus Real-Time PCR System (Applied Biosystems, CA, USA) in a 20 µl reaction mixture using a SensiFASTTM SYBR® kit (Bioline Reagent Ltd., UK) following a manufacturer's protocol. The reaction was performed at 95°C for 2 min, then 40 cycles of 95°C for 5 s, annealing temperature of each gene (see Table 1) for 10 s and 72°C for 10 s, followed by a dissociation curve step. The relative expression levels of the target genes were calculated by the $2^{-\Delta\Delta Ct}$ method. Relative mRNA levels were normalized to the 28S rRNA housekeeping gene and fold changes were expressed in relation to 4 months old control levels.

Table 1 Specific primers sequence for qrtRT-PCR. *Kiss1*, *Gnrh1*, and *Ar* are genes encoding kisspeptin, gonadotropin releasing hormone and androgen receptor, respectively.

Target Genes		Primer sequences (5'–3')	Product size	Accession no.	Annealing temp (°C)
Kiss1	forward	TGGCACCTGTGGTGAACCCTGAAC	202	NM_181692.1	60
	reverse	ATCAGGCGACTGCGGGTGGCACAC			
Gnrh1	forward	GCCGCTGTTGTTCTGTTGACT	234	NM_012767.2	56
	reverse	TTCCTCTTCAATCAGACGTTCC			
Ar	forward	GGGGCAATTCGACCATATCTG	278	NM_012502.1	58
	reverse	CCCTTTGGCGTAACCTCCCTT			
S28RNA	forward	GGCCGAAACGATCTCAACCT	248	NM_001106629.1	
	reverse	GCCACCGTCCTGCTGTCTAT			

Hormone assays

Blood samples were separated by centrifugation at 1600 xg for 20 min at 4 °C, blood sera were isolated and kept frozen at -20 °C until LH and testosterone levels assayed. Serum LH and testosterone levels were measured by Rat LH ELISA Kit no. CSB-E12654r (Cusabio, Hubei, China) and Testosterone ELISA Kit (ab108666, Abcam, MA, USA), respectively. Intra-assay coefficients of variation were 8.05% for LH and 6.52% for testosterone. The limits of detection (LOD) of the assay were 0.3 mIU/ml for LH and 0.2 ng/ml for testosterone.

Statistical analysis

All data are presented as mean ± SEM. One-way ANOVA with LSD post-hoc test was used to determine the difference of means. The SPSS software program (version 22.0, SPSS Inc., IL, USA) was used for the analysis. Significance levels were set at $p < 0.05$.

4. Results

Serum LH and testosterone levels

Serum LH levels was significantly decreased in 8 and 12 months old rats compared with the 4 months old (Fig 1A). The pattern of changes of serum testosterone levels was similar to that of the serum LH levels, that is, the lowering was initiated at 8 months old (1.282±0.311 ng/ml); however, the significant reduction was detected only at 12 months old (0.660±0.160 ng/ml) compared with the 4 months old (2.375± 0.576 ng/ml) (Fig 1B).

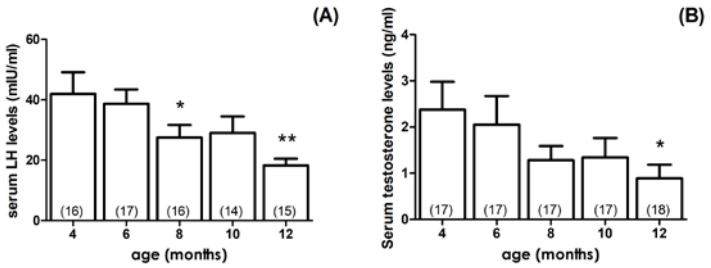

Fig. 1 Serum LH (A) and testosterone (B) levels of male rats at 4, 6, 8, 10 and 12 months old. Number in the parenthesis in each bar indicates the number of rats used in each month. * and ** represent $p < 0.05$ and 0.01 compared with the 4 months old rats.

mRNA expression levels of Kiss1, Gnrh1 and Ar genes in hypothalamus

In the same line with a decline of serum LH levels, *Gnrh1* mRNA levels in PoA were significantly decreased in 8 and 12 months old rats compared with the 4 months old rats (Fig 2A). The decrease in *Kiss1* mRNA levels in AVPV was initiated at 8 months old, but significant at 12 months old (Fig 2B). No significant changes of *Kiss1* mRNA levels in ARC were detected throughout those age range of rats (data not shown).

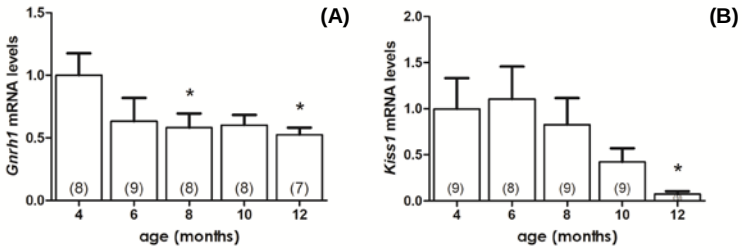

Fig. 2 mRNA levels of *Gnrh1* gene in the PoA (A) and *Kiss1* gene in the AVPV (B) of male rats at the age of 4, 6, 8, 10 and 12 months old. Number in the parenthesis in each bar indicates the number of rats used in each month. * represents $p < 0.05$ compared with the 4 months old rats.

A significant decrease in *Ar* mRNA levels in AVPV (Fig 3A) and ARC (Fig 3B) were started at 8 and 10 months old of rats, respectively.

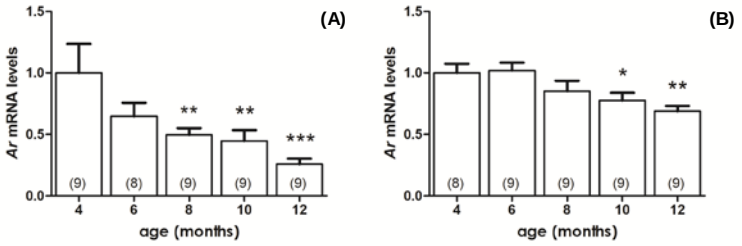

Fig. 3 mRNA levels of *Ar* gene in the AVPV (A) and ARC (B) of male rats at the age of 4, 6, 8, 10 and 12 months old. Number in the parenthesis in each bar indicates the numbers of rats used in each month. *, ** and *** represent $p < 0.05$, 0.01 and 0.001 compared with the 4 months old rats.

5. Discussion

Reproductive aging is associated with the loss of reproductive function and dysregulation of the hypothalamic-pituitary-gonadal axis (Golan, Scovell and Ramasamy, 2015). In this study, we used a significant decrease in serum testosterone level as an indicator of the reproductive aging of male Sprague-Dawley rats, and it can be detected when the rats were at the middle age (12 months old). Our results are in the same line with the previous studies in Wistar rats (Sokanovic et al., 2014) and Halan Sprague-Dawley rats (Wu, Lin and Gore, 2009). Although previous studies indicated that an androgen deficiency is cause by the deterioration of HPT axis (Golan, Scovell and Ramasamy, 2015; Gruenewald et al., 1994; Gruenewald et al., 2000), and the decline of GnRH secretion from the hypothalamus plays a key role in reproductive aging (Kermath and Gore, 2012; Yin and Gore, 2006), those studies looked at each level of the HPT axis, not the whole complex. From our results, we proposed that the decrease in testicular testosterone levels was attributed to the initiation of changes (at 8 months old of rat or before reaching the middle-aged stage) at the higher levels of the HPT axis. Straightforwardly, that is, *Kiss1* mRNA expression level at hypothalamic AVPV which was non-significantly reduced consequently led to a significant reduction of *Gnrh1* mRNA level in hypothalamic PoA, and a significantly decreased LH production by the anterior pituitary gland. We also hypothesized that the reduction of serum testosterone levels was caused by a dysfunction of the negative feedback system of the androgen to AR (Haji et al., 1981) because *Ar* expression was down-regulated at 8 months old in AVPV and 10 months old in ARC of hypothalamus. Similarly, Haji et al. (1981) reported that androgen binding with cytosol AR was decreased when the rats are at 300-330 days old compared with 90-100 days old.

However, our results contradict with previous studies denoting that the reproductive aging was initiated at the lower level of the HPT axis or at the testicular level (Tenover et al., 1987; Zirkin et al., 1993). Besides, those researchers used castrated male rats as subjects of an androgen deficient studies (Daniel, Winsauer and Moerschbaecher, 2003; Vanderschueren et al., 2000). In opposite to our study, Munetomo et al. (2015) determined age-induced changes in mRNA expression levels of sex-steroid receptors comparing between 3 (young age), 12 (middle age), and 24 (age) months old rats and found that *Ar* mRNA expression levels were increased in the hypothalamus of the middle-aged and aged-rats. Thus, it might be possible that if we assess the *Ar* mRNA expression profile of our male rats up to 24 months old, we might observe the increase.

Taken together from the results of our study, it indicates that reproductive aging in male Sprague Dawley rats occurs earlier at hypothalamus and pituitary levels and it is, thereafter, evident at the testicular level at 12 months old of rats. This is the first study to depict that the reproductive aging is initiated since the males enter their middle age. Thus, the prevention of any aging related diseases will be effective if it is performed before that time.

6. Conclusion

Male reproductive aging, indicating by a significant reduction of serum testosterone levels, occurs during their middle-age (12 months old) by the initiative deterioration of the higher center (both hypothalamus and pituitary gland) of the HPT axis at 8 months old.

Acknowledgements

This study was supported by the Grant for International Research Integration: Chula Research Scholar, Ratchadaphiseksomphot Endowment Fund (to S. Malaivijitnond) and the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund).

References

Cherrier, M. M., Asthana, S., Plymate, S., Baker, L., Matsumoto, A. M., Peskind, E., . . . Craft, S. (2001). Testosterone supplementation improves spatial and verbal memory in healthy older men. *Neurology*, *57*, 80-88.

Daniel, J. M., Winsauer, P. J., & Moerschbaecher, J. M. (2003). Castration in rats impairs performance during acquisition of a working memory task and exacerbates deficits in working memory produced by scopolamine and mecamylamine. *Psychopharmacology*, *170*, 294-300.

Golan, R., Scovell, J. M., & Ramasamy, R. (2015). Age-related testosterone decline is due to waning of both testicular and hypothalamic-pituitary function. *The Aging Male*, *18*, 201-204.

Gruenewald, D. A., Naai, M. A., Hess, D. L., & Matsumoto, A. M. (1994). The brown norway rat as a model of male reproductive aging: evidence for both primary and secondary testicular failure. *Journal of Gerontology, 49*, B42-B50.

Gruenewald, D. A., Naai, M. A., Marck, B. T., & Matsumoto, A. M. (2000). Age-related decrease in hypothalamic gonadotropin-releasing hormone (GnRH) gene expression, but not pituitary responsiveness to GnRH, in the male Brown Norway rat. *Journal of Andrology, 21*, 72-84.

Haji, M., Kato, K. I., Nawata, H., & Ibayashi, H. (1981). Age-related changes in the concentrations of cytosol receptors for sex steroid hormones in the hypothalamus and pituitary gland of the rat. *Brain Research*, *204*, 373-386.

Harman, S. M., Metter, E. J., Tobin, J. D., Pearson, J., & Blackman, M. R. (2001). Longitudinal effects of aging on serum total and free testosterone levels in healthy men. *The Journal of Clinical Endocrinology & Metabolism*, *86*, 724-731.

Jin, J.-M., & Yang, W.-X. (2014). Molecular regulation of hypothalamus–pituitary–gonads axis in males. *Gene, 551*, 15-25.

Kermath, B. A., & Gore, A. C. (2012). Neuroendocrine control of the transition to reproductive senescence: Lessons learned from the female rodent model. *Neuroendocrinology, 96*, 1-12.

Lv, W., Du, N., Liu, Y., Fan, X., Wang, Y., Jia, X., Hou, X. and Wang, B. (2016) Low testosterone level and risk of Alzheimer's disease in the elderly men: a systematic review and meta-analysis. *Molecular neurobiology*, *53*, 2679-2684.

Morley, J. E., & Perry, H. M., III. (2000). Androgen deficiency in aging men: role of testosterone replacement therapy. *The Journal of Laboratory and Clinical Medicine*, *135*, 370-378.

Munetomo, A., Hojo, Y., Higo, S., Kato, A., Yoshida, K., Shirasawa, T., . . . Kawato, S. (2015). Aging-induced changes in sex-steroidogenic enzymes and sex-steroid receptors in the cortex, hypothalamus and cerebellum. *The Journal of Physiological Sciences*, *65*, 253-263.

Oakley, A. E., Clifton, D. K., & Steiner, R. A. (2009). Kisspeptin signaling in the brain. *Endocrine Reviews, 30*, 713-743.

Paxinos, G., & Watson, C. (2005). *The Rat Brain in Stereotaxic Coordinates - The New Coronal Set* (5th Edition ed.).

Poletti, A., Rampoldi, A., Piccioni, F., Volpi, S., Simeoni, S., Zanisi, M., & Martini, L. (2001). 5Alpha-reductase type 2 and androgen receptor expression in gonadotropin releasing hormone GT1-1 cells. *Journal of Neuroendocrinology*, *13*, 353-357.

Rosano, G. M. C., Sheiban, I., Massaro, R., Pagnotta, P., Marazzi, G., Vitale, C., . . . Fini, M. (2006). Low testosterone levels are associated with coronary artery disease in male patients with angina. *International Journal of Impotence Research*, *19*, 176.

Seeburg, P. H., Mason, A. J., Stewart, T. A., & Nikolics, K. (1987). The mammalian GnRH gene and its pivotal role in reproduction. *Recent Progress in Hormone Research*, *43*, 69-98.

Smith, J. T., Dungan, H. M., Stoll, E. A., Gottsch, M. L., Braun, R. E., Eacker, S. M., . . . Steiner, R. A. (2005). Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse. *Endocrinology, 146*, 2976-2984.

Sokanovic, S. J., Janjic, M. M., Stojkov, N. J., Baburski, A. Z., Bjelic, M. M., Andric, S. A., & Kostic, T. S. (2014). Age related changes of cAMP and MAPK signaling in Leydig cells of Wistar rats. *Experimental Gerontology, 58*, 19-29.

Tenover, J. S., Matsumoto, A. M., Plymate, S. R., & Bremner, W. J. (1987). The effects of aging in normal men on bioavailable testosterone and luteinizing hormone secretion: response to clomiphene citrate. *The Journal of Clinical Endocrinology & Metabolism*, *65*, 1118-1126.

Tilbrook, A. J., & Clarke, I. J. (2001). Negative feedback regulation of the secretion and actions of gonadotropin-releasing hormone in males. *Biology of Reproduction, 64*, 735-742.

Vanderschueren, D., Vandenput, L., Boonen, S., Van Herck, E., Swinnen, J. V., & Bouillon, R. (2000). An aged rat model of partial androgen deficiency: prevention of both loss of bone and lean body mass by low-dose androgen replacement. *Endocrinology*, *141*, 1642-1647.

Veldhuis, J. D., Urban, R. J., & Dufau, M. L. (1992). Evidence that androgen negative feedback regulates hypothalamic gonadotropin-releasing hormone impulse strength and the burst-like secretion of biologically active luteinizing hormone in men. *The Journal of Clinical Endocrinology & Metabolism, 74*, 1227-1235.

Vermeulen, A., & Kaufman, J. M. (1995). Ageing of the hypothalamo-pituitary-testicular axis in men. *Hormone Research*, *43*, 25-28.

W Vale, C Rivier, a., & Brown, M. (1977). Regulatory peptides of the hypothalamus. *Annual Review of Physiology, 39*, 473-527.

Wu, D., Lin, G., & Gore, A. C. (2009). Age-related changes in hypothalamic androgen receptor and estrogen receptor alpha in male rats. *Journal of Comparative Neurology, 512*, 688-701.

Yin, W., & Gore, A. C. (2006). Neuroendocrine control of reproductive aging: roles of GnRH neurons. *Reproduction, 131*, 403-414.

Zirkin, B. R., Santulli, R., Strandberg, J. D., Wright, W. W., & Ewing, L. L. (1993). Testicular steroidogenesis in the aging brown Norway rat. *Journal of Andrology*, *14*, 118-123.