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SHORT COMMUNICATION Clinical Case Report

Acute testicular atrophy in an active Thoroughbred stallion

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Abstract

A Thoroughbred stallion was used as an active stallion for ten years, however, the number and motility of sperm dramatically decreased and azoospermia occurred at 16 years of age. Circulating luteinizing hormone (LH) was high at 16 years of age compared to the levels before the occurrence of these symptoms. Testicular endocrine function was examined by human chorionic gonadotropin (hCG) administration. No increase in circulating testosterone was detected at 16 years of age, whereas two peaks of testosterone were observed with hCG treatment at 12 years of age. The number of Leydig cells in the testes increased, but the LH receptor was not detected in Leydig cells. These results demonstrated that acute testicular atrophy occurred within one year.

Key Words: Azoospermia, Testicular atrophy, Thoroughbred stallion

Testicular atrophy, which includes oligospermia and azoospermia, is one of the causes of infertility in stallions[14]. Previous studies showed that testicular dysfunction was induced by various factors including as trauma[4], toxin[4,14], infection[6,14], thermal effect[6], endocrine disturbance[14], drug treatment[6], age[1,13], neoplasm[4], and torsion[4]. However, in most cases, the exact mechanisms by which testicular atrophy was induced were not known and there are no effective treatments for recovery of testicular dysfunction[1,5,9,12,14].

In the present study, testicular function during acute testicular atrophy in an active Thoroughbred stallion (stallion A) was analyzed by clinical observation, endocrine examination, and histological and immunohistochemical methods.

Stallion A was an active stallion for ten years from six years of age (2004) to 15 years of age (2013). The stallion was used in Hokkaido,

Y. Toishi and N. Tsunoda contributed equally to this study.

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Japan for nine years from 2004 to 2013 except 2005. The stallion was used for one year (2005) in the United Kingdom (UK). In Japan, the number of matings each year was 32 to 164 and the total number of matings was 890. The number of matings and conception rates in Japan are shown in Table 1. No reproductive abnormalities were observed in the stallion up to 15 years of age (2013). At the first mating in the eleventh breeding season, at 16 years of age (2014), a very small number of sperm without motility was found in the dismount semen, although libido and mating behavior were normal.

The size (length, height and width) of testes in stallion A were measured and the results are shown in Table 2. At 15 years of age (2013), the length, height and width of both testes in the stallion were similar to those at 14 years of age (2012), indicating that the sizes of testes were still in the normal range. However, the sizes of both testes were decreased at 16 years of age (2014) compared with those at 14 years of age (2012) (Table 2). At 16 years of age (2014), both testes of the stallion were atrophic and flabby on palpation. The testes of the stallion were examined by ultrasound echography (Prosound α-10, Aloka, Tokyo, Japan) and the results are shown in Fig. 1. Rete testes in the parenchyma and stock of sperm in the epididymis were not observed. There was no abnormality in the vascular system (Fig. 1).

Morphology of spermatozoa in the dismount semen of stallion A was examined using a sperm motility analysis system (IVOS, Hamilton Thorne Research, Beverly, MA, USA) from 11 years of age (2009) to 15 years of age (2013). The percentage of normal spermatozoa in the dismount semen was in the normal range, 78% ~ 91%, indicating that testicular function was normal until 15 years of age (2013) (data not shown). In clinical observation, the stallion showed nervous behavior when the testes were palpated at 15 years of age (2013). At 16 years of age (2014), the stallion mated with six mares and dismount semen was collected. In each mating, libido and ejaculation behavior were normal, though the sizes of both testes had decreased (Table 2). At the first mating at 16 years of age (2014), the spermatozoa were intact, but the number of sperm in the dismount semen was very small. Thereafter, the number of sperm gradually decreased and finally no sperm was found in the dismount semen at the sixth mating at 16 years of age (2014) (data not shown).

Table 1. Number of matings and conception rates of the stallion from 6 years of age (2004) to 15 years of age (2013)

Year	Age	Number of matings	Conception rate (%)
2004	6	144	70.8
2005	7	–	–
2006	8	122	56.6
2007	9	91	67.0
2008	10	84	75.0
2009	11	43	81.4
2010	12	32	59.4
2011	13	164	75.0
2012	14	145	66.2
2013	15	65	78.5

There are no data in 2005 because the stallion was used for one year (2005) in the United Kingdom.

The testes of the stallion were examined histopathologically after orchiectomy in December 2014 at 16 years of age. Orchiectomy was performed under general anesthesia that was induced with detomidine hydrochloride (Virbac, Milperra, Australia), diazepam (Takeda Pharmaceutical Company Limited, Osaka, Japan) and ketamine (Daiichi Sankyo Propharma, Tokyo, Japan) and maintained with isoflurane (Intervet, Tokyo, Japan). Photos of the testes after orchiectomy are shown in Fig. 2. The parenchyma of the testes was soft and flabby, and the tunica albuginea had become thicker (Fig. 2). The sizes of the testes were much smaller than those of an intact adult Thoroughbred stallion (Table 2).

A part of the testicular parenchyma was taken from the center of each testes. Samples of the testes were fixed in 10% buffered formalin, dehydrated, and embedded in paraffin. The

Table 2. Testicular sizes of the stallion from 14 years of age (2012) to 16 years of age (2014)

Date	Age	Left testis				Right testis			
		Length (cm)	Height (cm)	Width (cm)	Weight (g)	Length (cm)	Height (cm)	Width (cm)	Weight (g)
2012/1/23[a]	14	9.8	7.7	7.2	–	9.4	7.8	7.3	–
2013/1/22[a]	15	9.0	6.0	6.2	–	10.0	6.0	5.4	–
2014/2/15[b]	16	6.7	3.4	4.5	–	6.2	3.7	2.5	–
2014/12/16[c]	16	7.0	4.5	2.8	67.9	7.5	4.8	3.0	74.5

a) Testicular size was measured by scrotal calipers.
b) Testicular size was measured by ultrasound echography.
c) Testicular size and weight were measured after orchiectomy.

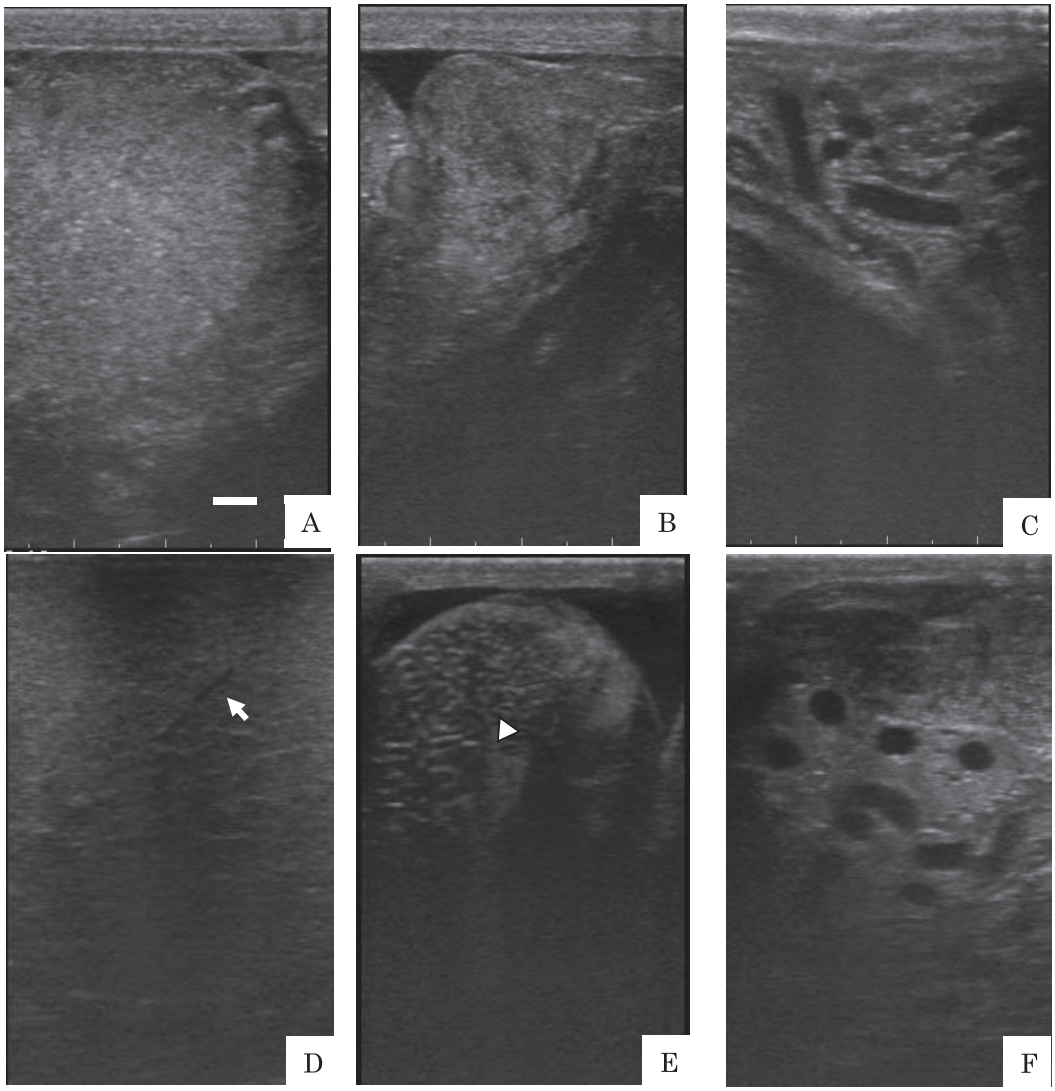


Fig. 1. Ultrasonographic appearances of the testes of a Thoroughbred stallion (stallion A) at 16 years of age (2014) (A to C) and an intact adult Thoroughbred stallion (22 years of age) (D to F). A, D: Parenchyma of the testes. Rete testes (arrow) in the parenchyma was not observed in A. B, E: Cauda epididymis. Stock of sperms (arrowhead) in the epididymis was not observed in B. C, F: The vascular system was normal. Scale bar in panel A represents 1 cm.

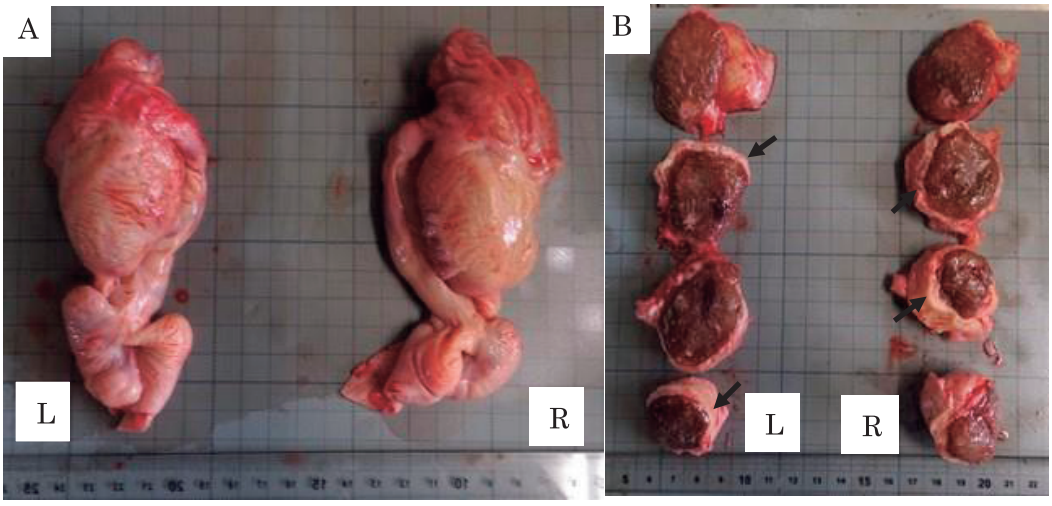


Fig. 2. Photos of both testes of a Thoroughbred stallion (stallion A) at 16 years of age (2014) after orchiectomy in December 2014 (A) and cross section of the testes (B). The parenchyma was soft and flabby. The tunica albuginea had become thicker (arrow in panel B). L and R represent left and right testes.

paraffin-embedded testes were serially sectioned at 4 μm in thickness, and serial sections were treated with xylene and graded ethanol and then stained with hematoxylin and eosin and Masson trichrome stain. Testes of an adult Thoroughbred stallion that died in December at the age of 21 year due to arteriorrhexis were used for a control.

In histological observation, the testes of the control stallion had seminiferous tubules showing complete spermatogenesis including spermatogonia, spermatocytes, spermatids and spermatozoa within the lumen (Fig. 3A, C). On the other hand, in the testes of stallion A, the layer of seminal epithelium in diffuse seminiferous tubules was thin, and there were tubules with arrested spermatogenesis (Fig. 3B, D). Sertoli cells within these tubules had large empty intracytoplasmic vacuoles (Fig. 3B, D). Leydig cells in the testes were swollen, and multilobular interstitial fibrosis was observed (Fig. 3B, D). The amount of collagen fibers was increased (Fig. 3F).

For immunohistochemical examination, testicular tissues were immune-stained against the equine luteinizing hormone (LH) receptor. Testicular paraffin sections were rinsed with 0.05% Triton-X 100 in PBS, and non-specific binding sites were blocked using 10% normal goat serum and 0.05% Tween 20 in PBS before incubation with a primary antibody. Epitope retrieval was carried out using a microwave (15 min in citrate buffer, pH 6.0). After cooling down to room temperature, the sections were incubated overnight at 4°C with an anti-equine LH receptor antibody (rabbit polyclonal antibody, 1 : 20 dilution, Biorbyt, Cambridge, UK). The sections were rinsed in distilled water, followed by treatment with a biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) by the ABC method with a commercially available kit (Vector Laboratories). Immunostaining using a single primary antibody was visualized using 3, 3'-diaminobenzidine tetrahydrochloraide as a substrate (Sigma-Aldrich, St Louis, MO, USA) and counterstaining with hematoxylin. The results clearly showed that there was no expression of the LH receptor in Leydig cells in the testes of stallion A, whereas expression of the LH receptor was observed in Leydig cells in testes of the control adult Thoroughbred stallion (Fig. 3G, H).

To evaluate the endocrine function of the hypothalamo-hypophysial-testicular axis, basal concentrations of serum LH and testosterone were measured in stallion A during the breeding and non-breeding seasons at 14 years of age (2012), before testicular atrophy had developed, and at 16 years of age (2014), after testicular atrophy

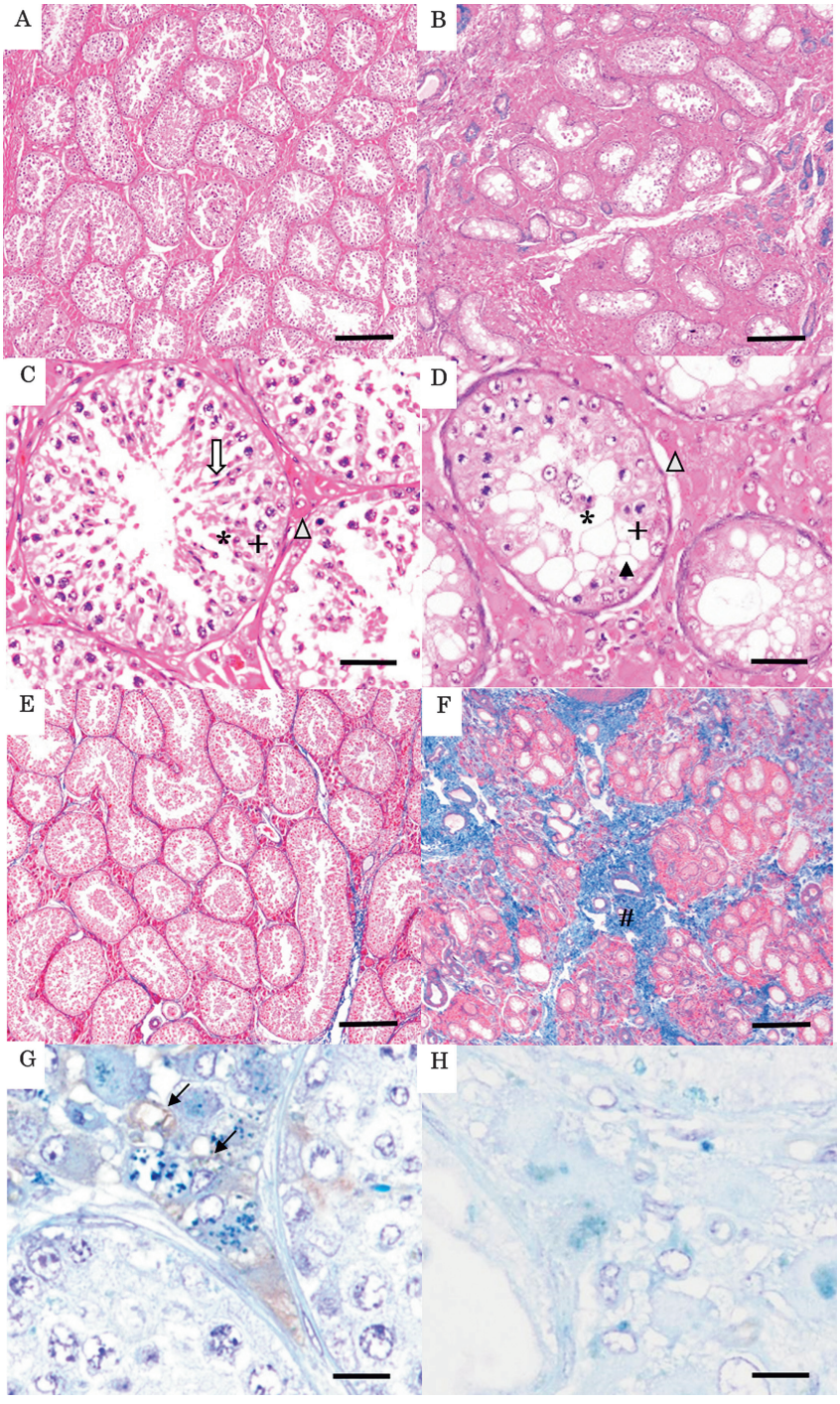


Fig. 3. Histological (A~F) and immunohistchemical (G, H) observations of the testes of an intact adult Thoroughbred stallion (A, C, E, G) and Thoroughbred stallion A (B, D, F, H). Complete spermatogenesis including spermatocytes (C, +), spermatid (C, *) and spermatozoa (C, white arrow) was observed within the lumen of the intact adult Thoroughbred stallion (A, C). Spermatogenesis was arrested in stallion A (B, D). Sertoli cells within tubules had large empty intracytoplasmic vacuoles in stallion A (D, black arrowhead). Leydig cells (C, D, white arrowhead) were swollen. The amount of collagen fibers was increased (F, #). In immunohistchemical sections, LH receptors were observed in Leydig cells in the testes of an intact adult Thoroughbred stallion (G, black arrow) but not in Leydig cells in the testes of stallion A (H). A~D: Hematoxylin and eosin stain. E, F: Masson trichrome stain. G, H: immune-stain against the equine LH receptor. Bars in panels A, B, E and F represent 200 μm, bars in panels C and D represent 40 μm, and bars in panels G and H represent 13.3 μm.

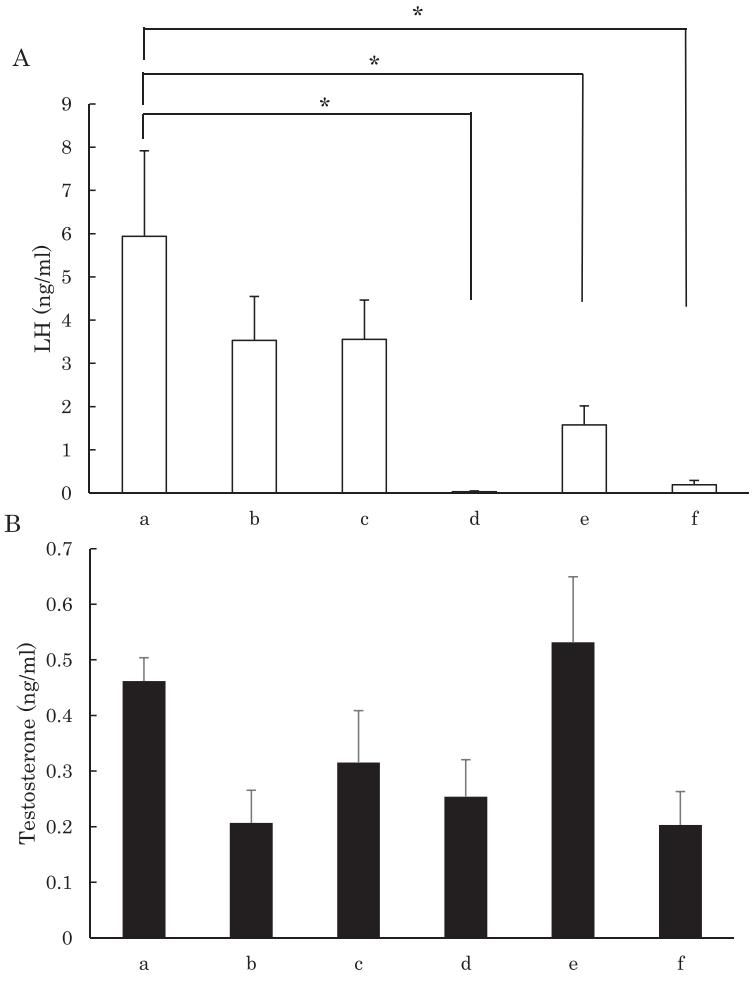


Fig. 4. Basal concentrations of circulating luteinizing hormone (LH) (A) and testosterone (B) in a Thoroughbred stallion (stallion A) during the breeding and non-breeding seasons and in the intact adult Thoroughbred stallion. a: Stallion A after the development of testicular atrophy in the breeding season (n = 4). b: Stallion A after development of testicular atrophy in the non-breeding season (n = 3). c: Stallion A before the development of testicular atrophy in the breeding season. d: Stallion A before the development of testicular atrophy in the non-breeding season (n = 6). e: Intact Thoroughbred stallions in the breeding season. f: Intact Thoroughbred stallions in the non-breeding season (n = 6). Results are expressed as means ± standard error of the mean. *$P < 0.05$.

had developed. The results are shown in Fig. 4. Serum concentrations of LH and testosterone in intact adult Thoroughbred stallions during the breeding and non-breeding seasons are shown in Fig. 4.

Blood samples were collected from the jugular vein of stallion A during the breeding season (group a, n = 4, April to July at 16 years of age) and during the non-breeding season (group b, n = 3, September to November) after testicular atrophy had developed. Serum samples were harvested and stored at −20°C until assayed for LH and testosterone. Blood samples were also collected from stallion A before the development of testicular atrophy in the breeding season (group c, n = 5, April at 11 to 15 years of age) and in the non-breeding season (group d, n = 5, October at 11 to 15 years of age). Blood samples were also collected from intact adult Thoroughbred stallions from 14 to 16 years of age during the breeding season (group e, n = 6, April) and the non-breeding season (group f, n = 6, October) as an intact control. Serum concentrations of LH were determined by using the equine homologous

double-antibody radioimmunoassay (RIA) system as described previously[3]. Intra-assay and inter-assay coefficients of variance were 12.6% and 15.1%, respectively. Serum concentrations of testosterone were measured by a chemiluminescent enzyme immunoassay (PATHFAST, LSI Medience Corporation, Tokyo, Japan) as descrived previously[10]. The intra-assay coefficients of variation were 8.9% at 0.87 ng/ml and 5.6% at 11.60 ng/ml. One-way ANOVA and Tukey's multiple comparison test were performed to detect significant changes in the concentrations of hormones using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

As shown in Fig. 4, serum concentrations of LH in group a at 16 years of age (2014), after testicular atrophy had developed, tended to be higher than those at 14 years of age (2012), before testicular atrophy had developed. Serum concentrations of LH in group a were significantly higher than those in groups d, e and f (Fig. 4A). Although serum concentrations of testosterone in groups a, c and e tended to be higher than those in groups b, d and f, there was no significant difference among the groups (Fig. 4B). There was no significant difference in serum concentrations of testosterone among any of the groups (Fig. 4B). Our previous studies demonstrated that there was a clear seasonal changes in circulating LH and testosterone in Thoroughbred stallions[3,8]. The highest circulating concentrations of LH and testosterone were observed in the breeding season, and the lowest levels were observed during the non-breeding season. The present study showed that there were clear seasonal changes in circulating LH in the intact adult Thoroughbred stallions and stallion A at 14 years of age but not in stallion A at 16 years of age. These results indicate that the negative feedback relationship between the hypothalamo-hypophysial-testicular axis by testosterone had less strength in stallion A at 16 years of age than that in stallion A at 14 years of age and in the intact stallions.

The endocrine function of the testis in stallion A was further evaluated by treatment with 10,000 IU of human chorionic gonadotropin (hCG)[10,11]. The experiments were carried out at 12 years of age (2010), before testicular atrophy had developed, and at 16 years of age (2014), after testicular atrophy had developed, and the results of both experiments are shown in Fig. 5. Blood samples were taken from the jugular vein just before administration, each hour for six hours and every day for seven days after administration of hCG.

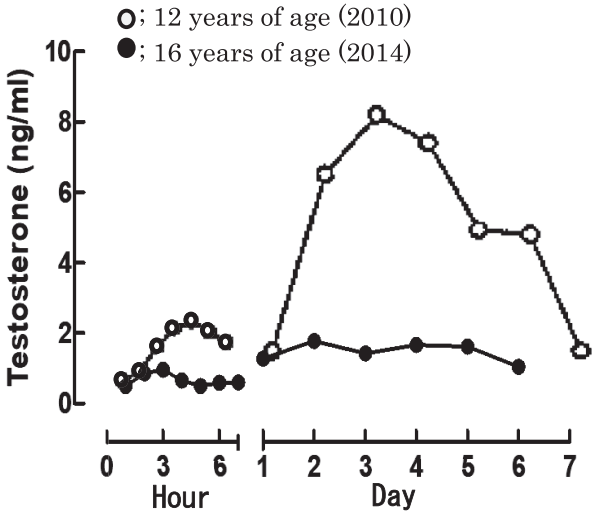


Fig. 5. Changes in circulating testosterone in Thoroughbred stallion A after a single administration of 10,000 IU of human chorionic gonadotropin (hCG) at 12 years of age (2010) and 16 years of age (2014).

Following intramuscular administration of hCG in stallion A at 12 years of age (2010), serum concentration of testosterone was increased at one hour and reached the first peak at four hours, followed by a gradual decline until six hours after hCG administration. The serum concentration of testosterone began to increase again from two days and reached the second peak at three days after hCG administration, followed by a gradual decline until seven days after hCG administration. On the other hand, an increase in the serum concentration of testosterone was not observed after intravenous administration of hCG in stallion A at 16 years of age (Fig. 5), indicating that Leydig cells in the

testes of stallion A at 16 years of age were less sensitive to hCG stimulation than were Leydig cells at 12 years of age.

The present study clearly demonstrated that acute testicular atrophy occurred in stallion A. The last mating of stallion A at 15 years of age was on June 2, 2013 and the first mating at 16 years of age was on February 15, 2014, indicating that acute testicular atrophy occurred within nine months. Results of the endocrine examination also suggested that the negative feedback regulation of LH secretion by testosterone clearly changed within two years. Circulating LH level was higher at 16 years of age (2014), after testicular atrophy had developed, than at 14 years of age (2012), before testicular atrophy had developed. These results suggested that the negative feedback effect of testosterone on the hypothalamus and pituitary for suppressing kisspeptin, gonadotropin-releasing hormone and LH was reduced within two years in stallion A. In addition, endocrine function of Leydig cells in the testes for secreting testosterone in response to hCG had dramatically declined at 16 years of age (2014) due to loss of LH receptors in Leydig cells. There has been no report of loss of LH receptors in Leydig cells of equine testes during the process of testicular atrophy. However, a decreased number of LH receptors was found in aged rats without a change in the number of Leydig cells[2].

The testes of stallion A secreted testosterone in response to hCG stimulation, and the level of circulating testosterone at the second peak was four-times higher than that at the first peak in stallion A at 12 years of age. Our previous study clearly demonstrated that a single administration of hCG in a Thoroughbred stallion induced two peaks of circulating testosterone, at six hours and two days after administration of hCG, and the level of circulating testosterone was much higher at the second peak than at the first peak[11]. The present study, therefore, clearly suggested that the endocrine function of Leydig cells of stallion A was intact at 12 years of age (2010).

In conclusion, the present study clearly demonstrated that azoospermia occurred in stallion A due to primary hypogonadism, though the exact mechanism is unknown. The present study also demonstrated that evaluation of the dismount semen is a useful method for rapid diagnosis of azoospermia in a stallion.

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