



NOTE

Theriogenology

LH and testosterone secretions in response to GnRH challenge in pubertal Japanese Black beef bulls with normal and abnormal semen

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ABSTRACT. Plasma luteinizing hormone (LH) and testosterone concentrations were examined in Japanese Black beef bulls with normal and abnormal semen in response to gonadotropin releasing hormone (GnRH) challenge at the start (10 months) and completion (20 months) of puberty. Bulls with normal semen had higher testosterone concentrations after GnRH treatment at 20 months than they did at 10 months, while LH concentrations did not differ between the two age groups. LH and testosterone concentrations were not different between bulls with normal and abnormal semen at 20 months. Thus, testosterone secretions in response to the GnRH challenge were higher for bulls with normal semen at pubertal completion compared to bulls at the start of puberty, but responsiveness of LH to GnRH and of testosterone to the LH increment was not altered in bulls with abnormal semen.

KEY WORDS: beef bull, GnRH challenge, LH, semen, testosterone

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Japanese Black is a dominant beef breed in Japan that produces highly marbled beef [9, 16]. A small number of Japanese Black bulls showing superior carcass traits in their progeny are selected as sires from many young candidates. Occasionally the sire candidates have abnormal semen that is infertile or subfertile [12, 17]. The causes of the aberrant semen are largely unknown. Recently, it was shown that plasma insulin-like growth factor-I (IGF-I), insulin-like peptide 3 (INSL3), and inhibin concentrations surrounding puberty were reduced in the Japanese Black beef bulls with abnormal semen while testosterone concentrations were not clearly decreased [18].

Serum concentrations of luteinizing hormone (LH) in beef bull calves at 5 months of age were lower for early maturing bulls in response to gonadotropin releasing hormone (GnRH) challenge than for late maturing animals [2]. Blood testosterone concentrations after the GnRH challenge increased during calthood (from 3.5 to 6.5 months of age) in beef bulls [3]. It has been suggested that incremental concentrations of serum testosterone after the GnRH challenge are enhanced in Brahman bulls from pre-pubertal to post-pubertal age [15]. In the post-pubertal Brahman bulls, the testosterone secretions after the GnRH challenge were lower in the winter, when the semen quality was deteriorated, than in spring and summer [4]. It is well known that testosterone secretion in response to LH is essential for normal spermatogenesis and functions of the accessory sex glands in bulls [14, 17]. However, the LH and testosterone responses to GnRH challenge in Japanese Black beef bulls before and after puberty have not yet been elucidated for bulls with abnormal semen. If the hormonal responses to the GnRH challenge are altered in young bulls with abnormal semen, testing those responses would be a useful method for screening and culling the abnormal bulls early in semen production stations. As the Japanese Black beef bulls are highly valuable, it would be profitable to identify infertile bulls at a young age by endocrine examination.

The objectives of the present study were to: (1) examine blood LH and testosterone concentrations in response to the GnRH challenge in Japanese Black beef bulls at the start and the completion of puberty; and (2) compare the hormonal secretions in response to the GnRH challenge between bulls having normal and abnormal semen at the pubertal completion.

Japanese Black beef bulls (n=21) in an experimental beef cattle station at the Hokubu Agricultural Institute, Hyogo Prefectural

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Table 1. Age (days) at the gonadotropin releasing hormone (GnRH) challenge, and semen volume, sperm concentration, motility, and rate of malformation at 18 months in bulls with normal semen and bulls with abnormal semen

	Age (days)	Semen volume (ml)	Sperm concentration ($10^8/\text{ml}$)	Motility (%)	Rate of malformation (%)
Normal 10 months (n=13)	311.5 \pm 1.8	4.3 \pm 0.2	8.0 \pm 0.5	80.0 \pm 0	8.6 \pm 0.9
Normal 20 months (n=4)	619.0 \pm 5.6	3.8 \pm 0.3	7.9 \pm 0.9	80.0 \pm 0	11.4 \pm 1.5
Malformation + low motility 20 months (n=4)	624.5 \pm 4.0	4.3 \pm 0.6	5.0 \pm 1.3	51.3 \pm 13.0	23.9 \pm 2.5

Data are expressed a mean \pm SEM.

Technology Center for Agriculture, Forestry and Fisheries in Japan were used for the present study. The bulls remained normal in appearance and healthy during all experiments. The experiments were conducted from October 2014 to December 2015. The bulls were kept under natural light in an open shelter (covered by a roof) and were provided with hay and concentrates to meet or exceed the Japanese Feeding Standard recommendations for beef bulls. The following experiments were approved by the Hokubu Agricultural Institute, Hyogo Prefectural Technology Center for Agriculture, Forestry and Fisheries. The procedures for the animal experiments complied with the guidelines for the Proper Conduct of Animal Experiments at Academic Research Institutions in Japan.

A GnRH analogue (fertirelin acetate; Conceral[®], Intervet, Tokyo, Japan; 50 $\mu\text{g}/100$ kg BW, IM) was given to bulls at 10 months (start of puberty, n=13), and at 20 months (completion of puberty) for bulls with normal semen (n=4) and abnormal semen (n=4). Blood samples were obtained at -0.5, 0 (just before the GnRH analogue treatment), 1, 2, 3, 4, 5 and 6 hr. Blood samples were collected from the jugular vein into heparinized tubes and immediately placed on ice. The blood was centrifuged at 1,700 g for 15 min at 4°C. The separated plasma was stored (-30°C) until assay.

The bulls body weight and scrotal circumference were recorded monthly from 8 to 20 months of age. Semen was collected from all the bulls weekly from 12 months until at least 18 months of age through the use of an artificial vagina. Two ejaculates were collected during each collection with a 5- to 10-min interval. The fresh semen was evaluated for volume (normal >3 ml), concentration (normal >500 million/ml), rate of sperm with highly progressive motility (normal $\geq 80\%$), and rate of morphological defects (normal <20%) [1, 13]. The morphological examination was performed using the Hemacolor stain set (Merck, Darmstadt, Germany). For each semen collection, 500 sperm were examined for morphological defects of the head, midpiece, and tail. According to the above criteria, bulls were classified as having either normal or abnormal semen. All the bulls with abnormal semen (n=4) had both low motility and a high rate of morphological defects in their sperm, while other bulls (n=17) had normal semen. Table 1 shows the age of the bulls (in days) at the GnRH challenge, semen volume, sperm concentration, motility and rate of morphological defects in the bulls with normal and abnormal semen.

Plasma LH concentrations were assayed by an enzyme immunoassay (EIA) described previously [5] using biotinylated bovine LH (AFP11743B, NIDDK, Bethesda, MD, U.S.A.) and anti-bovine LH antibody (Immundiagnostik AG, Bensheim, Germany). The minimum detection limit was 0.31 ng/ml, and the reliable detection limit was 0.31 to 40 ng/ml. The intra-assay and inter-assay CVs were 4.4% (n=4) and 14.7% (n=5), respectively. Plasma testosterone concentrations were measured by an EIA that was established in our laboratory [10, 11]. The minimum detection limit was 0.156 ng/ml, and the reliable detection limit was 0.156 to 20 ng/ml. The intra-assay and inter-assay CVs were 4.9% (n=4) and 14.8% (n=5), respectively.

We investigated the effects of age (month) and semen (bulls with normal semen at both 10 months and 20 months and bulls with abnormal semen at 20 months) on body weight and scrotal circumference by conducting a two-way analysis of variance (ANOVA) using the Generalized Linear Models (GLMs) procedure of SPSS ver. 24 software (IBM, Somers, NY, U.S.A.). Additionally, we investigated the effects of time (hr) and age (month) or semen (bulls with normal semen at 20 months and bulls with abnormal semen at 20 months) on plasma LH and testosterone concentrations by the ANOVA using the GLMs procedure of SPSS ver. 24 software. *Post-hoc* pairwise comparisons were made by the Bonferroni correction for differences between two time-points of the same semen group. The *post-hoc* pairwise comparisons were performed by the least significant difference test for differences between bulls aged 10 and 20 months with normal semen or between the bulls with normal semen and the bulls with abnormal semen at a specific time point. The data are expressed as mean \pm SEM. Differences were considered significant at $P < 0.05$.

Changes in body weight and scrotal circumference in the bulls with normal and abnormal semen (sperm malformation plus low motility) are shown in Fig. 1. There were significant effects of age and semen ($P < 0.0001$) on body weight, but no significant interaction was observed (Fig. 1A). The body weight gained constantly from 8 to 18 months of age ($P < 0.05$) but did not increase significantly between 18 and 20 months. There were no significant differences of body weight between the bulls with normal semen and the bulls with abnormal semen at any specific age. There were significant effects of age ($P < 0.0001$) and semen ($P < 0.05$) on scrotal circumference, but no significant interaction (Fig. 1B). The scrotal circumference increased constantly from 8 to 14 months of age ($P < 0.05$) but did not significantly increase thereafter until 20 months. No significant differences of scrotal circumference were observed between bulls with normal semen and bulls with abnormal semen at any specific age.

In the comparison between the bulls with normal semen at 10 and 20 months, there were significant effects of age ($P < 0.05$) and time ($P < 0.0001$) on the plasma LH and testosterone concentrations, but no significant interaction was observed between age and time (Fig. 2A and 2B). The LH concentrations increased ($P < 0.05$) from 0 hr (0 to 1, 2, 3, 4, and 5 hr after the GnRH treatment) and returned to the 0 hr value at 6 hr in both the 10- and 20-month age groups. The LH concentrations did not differ significantly

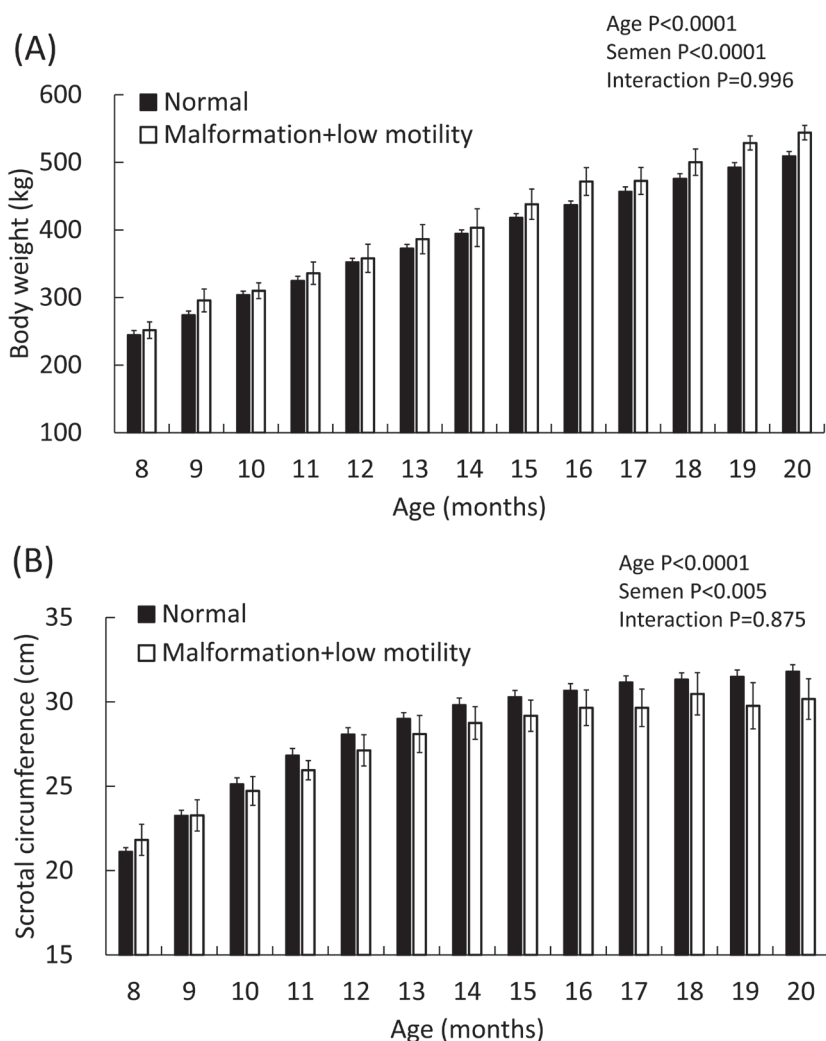


Fig. 1. Changes of body weight and scrotal circumference in Japanese Black beef bulls with normal semen (n=17) and bulls with abnormal semen (n=4; sperm malformation plus low motility). Data are expressed as the mean \pm SEM. The data of bulls with normal semen were combined of those from bulls treated with GnRH at 10 months (n=13) and 20 months (n=4).

between the two age groups. Rates of increment (peak concentrations/0 hr concentrations) of LH in the bulls at 10 and 20 months were 5.73 and 6.22, respectively. The testosterone concentrations increased ($P<0.05$) from 0 hr to 1, 2, 3, 4, 5 and 6 hr after the GnRH treatment in both 10- and 20-month age groups. The testosterone concentrations were significantly higher ($P<0.05$) in the bulls with normal semen at 20 months compared to those at 10 months at -0.5 , 1, 2, 3, 4, 5 and 6 hr. The rates of increment of testosterone in the bulls at 10 and 20 months were 4.33 and 3.43, respectively. There were no clear effects of season on LH and testosterone concentrations in the bulls with normal semen treated with GnRH at 10 months.

There were significant effects of time ($P<0.0001$) on plasma LH and testosterone concentrations in the comparison between the bulls with normal semen and abnormal semen at 20 months of age (Fig. 3A and 3B). However, effects of semen on LH and testosterone concentrations and the interaction between semen and time were not significant. The LH concentrations increased ($P<0.05$) from 0 hr to 1, 2 and 3 hr after the GnRH treatment in the bulls with abnormal semen and declined to the 0 hr value at 4 hr. The LH concentrations did not differ significantly between the bulls with normal semen and the bulls with abnormal semen during the 6.5 hr assay. The testosterone concentrations rose ($P<0.05$) from 0 hr to 1, 2, 3, 4, 5 and 6 hr after the GnRH treatment in the bulls with abnormal semen. There was no significant difference in testosterone concentrations between the bulls with normal semen and the bulls with abnormal semen at any time point. The rates of increment of LH and testosterone in the bulls with abnormal semen were 5.58 and 3.00, respectively.

It is well known that blood testosterone concentrations increase before and after puberty in bulls [10, 14]. However, information regarding changes in LH and testosterone secretions in response to GnRH challenge before and after puberty in Japanese Black beef bulls is not available. The results of this study show that the plasma testosterone concentrations were higher for bulls with

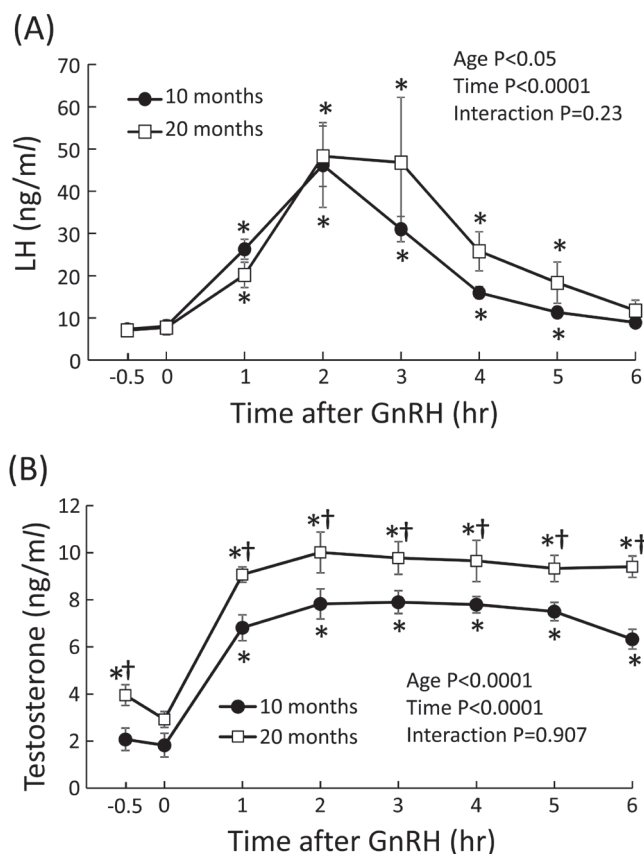


Fig. 2. Concentration changes in plasma LH (A) and testosterone (B) in response to GnRH challenge in Japanese Black beef bulls at 10 months of age ($n=13$) and bulls with normal semen at 20 months of age ($n=4$). Data are expressed as the mean \pm SEM. * $P < 0.05$, compared to the 0 hr value within a group. † $P < 0.05$, compared to bulls at 10 months at the same time-point.

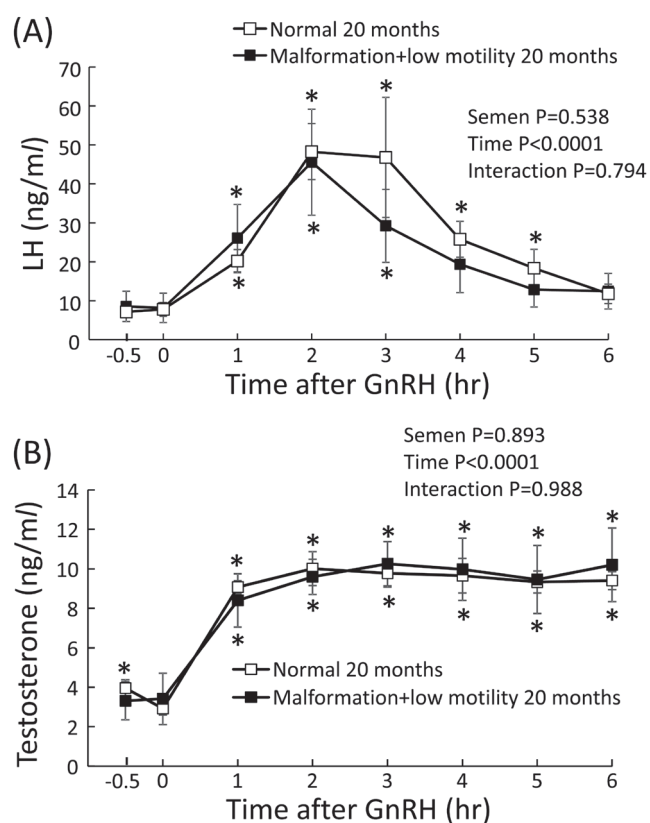


Fig. 3. Concentration changes in plasma LH (A) and testosterone (B) in response to GnRH challenge in Japanese Black beef bulls with normal semen at 20 months of age ($n=4$) and bulls with abnormal semen (malformation plus low motility of sperm) at 20 months of age ($n=4$). Data are expressed as the mean \pm SEM. * $P < 0.05$, compared to the 0 hr value within a group.

normal semen at the completion of puberty than for those at the onset of puberty at most of the time-points before (basal level) and after (enhanced level in response to endogenous LH increases) the GnRH challenge. On the other hand, the LH concentrations in response to the GnRH challenge did not differ between the pre-pubertal and post-pubertal bulls. These results may suggest that the secretion capacity of testosterone in Leydig cells, including the total number of the cells per bull, is augmented after the pubertal completion in conditions with or without LH increment in the beef bulls. Thus, it seems essential to use bulls at the same age, especially surrounding puberty, when comparing secretion capacity of testosterone between normal and abnormal reproductive functions. This study shows no clear effect of season on testosterone concentrations in the Japanese Black beef bulls with normal semen treated with GnRH at 10 months, while the testosterone secretions in the Brahman bulls were lower in the winter, when their semen quality had deteriorated, than in the spring [4]. It is unclear why seasonal effects on testosterone secretion were inconsistent between both studies, although different subspecies (*Bos indicus* vs *Bos taurus*) or location (Montana, U.S.A. vs Hyogo, Japan) of the bulls may have caused the difference in results.

In the current study, no clear differences were observed in plasma LH and testosterone concentrations between bulls with normal and abnormal semen (malformation plus low motility of sperm) at the same age after the GnRH challenge. Also, the rates of increments (peak value/0 hr value) of both hormones by the GnRH treatment did not differ between the bulls with normal and abnormal semen. These results indicate that the responsiveness of LH secretion to GnRH in the anterior pituitary and the responsiveness of testosterone secretion to LH in the testicular Leydig cells are not altered in the beef bulls with the semen aberration. In this context, we observed no marked differences in the previous study of plasma testosterone concentrations without hormonal treatments between the bulls with normal semen and the bulls with the malformation plus low motility of sperm from 4 to 24 months of age [18]. In the same report [18], clear reductions of plasma INSL3 concentrations were found in the bulls with the abnormal semen compared to the bulls with the normal semen. INSL3 is secreted from Leydig cells, as is the case with testosterone, and regulation of the secretion seems different from testosterone in male ruminants [5–8, 10, 14]. We infer that blood INSL3 concentration is superior to testosterone as a biomarker of testicular total Leydig cell volume [8].

In conclusion, the secretion capacity of testosterone before and after the GnRH treatment was augmented at the completion

of puberty in the Japanese Black beef bulls with normal semen while the LH secretion in response to the GnRH did not change. Responsiveness of LH secretion to the GnRH and of testosterone secretion to the LH increment was not altered in the beef bulls with the semen aberration and malformation plus low motility of sperm.

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