Anti-Müllerian Hormone (AMH) Profiles as a Novel Biomarker to Evaluate the Existence of a Functional Cryptorchid Testis in Japanese Black Calves

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Abstract. Anti-Müllerian hormone (AMH) and testosterone (T) profiles in blood were investigated before and after an hCG stimulation test to assess their sensitivity and specificity for the existence of a functional cryptorchid testis in Japanese Black calves. The hCG (3,000 IU) was administered on Day 0, and peripheral blood was collected on Days 0 (just before hCG injection), 5 and 7 in intact male calves (Intact; n=19), bilateral castrated calves (Castrated; n=17), unilateral cryptorchid calves, which abdominal testis could be extracted (Uni-crypto; n=9). Castration of a descended testis was carried in the Castrated and Uni-Crypto groups on Day -14. The AMH detectability and the optimum cut-off point for T levels using the receiver operating characteristic curve were verified to characterize the cryptorchid testis. AMH values on Day 0 were 21.1 ± 5.1 and 29.0 ± 7.5 ng/ml in the Intact and Uni-crypto groups, respectively (Mean ± SEM). AMH levels were under the detection limit in the Castrated group (i.e., < 0.006 ng/ml). T showed its peak levels on Day 5 in the Intact group (26.8 ± 4.2 ng/ml), while it remained low in the Castrated group (< 0.9 ng/ml) and did not show a significant difference in the Uni-crypto group. The detectable levels for AMH was 0.006 ng/ml, and the optimum cut-off point for T was 0.9 ng/ml; the sensitivity and specificity for evaluation of testicular descent into the scrotum were 1.0 for both the AMH and T levels. The detection rates in the Uni-crypto group using them were 1.0 and 0.57 for AMH on Day 0 and T on Days 5 or 7, respectively. In conclusion, plasma AMH profiles could be used as a novel biomarker to evaluate the existence of a functional cryptorchid testis in Japanese Black calves.

Key words: Anti-Müllerian hormone (AMH), Bovine cryptorchidism, hCG stimulation test, Receiver operating characteristic, Testosterone


Cryptorchidism is the failure of one or both testes to descend into the scrotum during the first trimester of pregnancy [1]. The plausible causes of cryptorchidism include insufficient secretion of insulin-like peptide 3 and/or testosterone (T) [1]. Cryptorchidism is most common in stallions, boars and some breeds of canine, but is rare in most other species [2, 3]. The rate of morbidity with cryptorchidism in cattle is 0.2% [4]. The internal body temperature that surrounds the intra-abdominal retained testes associated with cryptorchidism is higher in comparison with that in the scrotum; this compromises spermatogenesis [5].

Calves with a cryptorchid testis are commonly detected at castration [3]. If they have unilateral cryptorchid testes, the contralateral descended testes are extracted on the same day, and the cryptorchid testis is then usually looked for by laparotomy on another day. However, exteriorization of an abdominal testis is difficult [6].

Fattening bulls with cryptorchidism in the later growing stages usually result in bulls with a stout body type that exhibit masculine characteristics in the neck region and in their skull formation because their functional cryptorchid tissues synthesize and secrete T [7]. Moreover, this aggressive bulling results in a lowered quality of beef. It is necessary to distinguish whether bulls with a non-palpable testis in the scrotum have a functional or malfunctional cryptorchid testis or anorchia.

One of the endocrinological approaches to evaluation of the existence and functionality of a testis is the measurement of plasma T concentrations before and after an hCG stimulation. This test is based on the fact that Leydig cells of the testis are stimulated by luteinizing hormone (LH) to synthesize T [8]. In a similar manner, exogenous hCG exhibits LH-like effects. Functional Leydig cells synthesize T following hCG administration. This test has been validated for the bull [9], ram [10], boar [11], stallion [12] and human [13]. However, the profiles of plasma T concentrations after an hCG stimulation test have not been shown to be invariably similar, especially among prepubertal children [14] and bulls with cryptorchidism [15]. The profiles of plasma T concentrations after an hCG stimulation test have been comprehensively evaluated with
normally descended testes [16, 17]. However, the corresponding endocrinological profiles have not been thoroughly clarified with either unilaterally or bilaterally retained testes. The plasma T concentrations following experimental unilateral castration (with the other testis retained in the scrotum) remain to be clarified.

In human medicine, the existence and function of a cryptorchid testis has been investigated using the plasma profiles of anti-Müllerian hormone (AMH) [18]. AMH is primarily secreted from embryonic Sertoli cells and induces regression of the Müllerian ducts during early male sexual differentiation [19]. In addition, AMH plays critical regulatory roles in postnatal development, sexual maturation and reproductive function; for example, AMH inhibits the differentiation and proliferation of Leydig cell precursors and downregulates androgen steroidogenesis in mature Leydig cells in mice [2, 20]. However, no report has examined plasma AMH profiles associated with cryptorchidism in cattle.

This study was carried out to compare the sensitivity and specificity of plasma AMH profiles in comparison with T profiles before and after an hCG stimulation test to evaluate the existence of a functional cryptorchid testis after castration of the contralateral descended testis by receiver operating characteristics (ROC) analysis.

Materials and Methods

Animals

A total of 45 Japanese Black calves were enrolled from January 2009 to March 2010 in this study: 36 were normal male Japanese Black calves at 6 months of age. These normal calves were evaluated at the Experimental Field in University of Miyazaki, Japan. In the remaining 9 calves, one testis could not be palpated in the scrotum (5 left-side and 4 right-side nonpalpable testes) at 254.6 ± 34.9 days old (mean ± SEM, range of 185–523 days old). These calves belonged to private farms in Miyazaki Prefecture, Japan. After the end of this study, the undescended testes could be extracted from the abdomen by laparotomy and had a confirmed parenchyma and epididymis. Therefore, the cows were diagnosed with unilateral cryptorchidism.

Experimental design

The previously mentioned animals were assigned into 3 groups, intact male calves (Intact group, n = 19), bilateral castrated calves (Castrated group, n=17) and the unilateral cryptorchid calves (Uni-crypto group, n=9).

Bilateral castration in the Castrated group was performed surgically and was carried out at 14 days (Day -14) before starting the hCG stimulation test (Day 0) in male calves. In the Uni-crypto group, the testis descended in the scrotum was also castrated on Day -14.

Each male calf in the previously mentioned groups received a single intramuscular injection of 3,000 IU of hCG (Veterinary Puberogen, Novartis Animal Health, Tokyo, Japan).

Blood sampling

Blood samples were collected by jugular vein venipuncture into heparinized vacuum tubes on Day 0 immediately before hCG administration and on Days 5 and 7. Blood samples were centrifuged immediately at 4 C and 3,000 rpm for 20 min. The plasma was harvested and stored at –30 C until hormonal assay.

Hormonal assays

Since T levels in blood change with LH pulsatile secretion, they were measured before and after hCG injection. By contrast, AMH levels are not regulated by the hypothalamic-pituitary-gonadal axis [21], so they were measured only on Day 0 before hCG injection.

Plasma AMH concentrations on Day 0 were measured by ELISA (Active MIS/AMH ELISA, DSL-10-14400, Beckman Coulter, USA), which had been previously validated for cattle [22, 23]. Plasma T concentrations were measured using an Enzyme-Linked Fluorescent Assay (ELFA; VIDAS testosterone, Japan bioMerieux, Tokyo, Japan). For the assay validation of T, the addition-recovery test (added two different known concentrations of T, 1 ng and 5 ng/ml, measured in triplicate). Parallelisms between the dilution curves for blood in humans and bovines (each dilution point was measured in triplicate, Fig. 1) were investigated. The percentage of T recovered by this assay was 92.7% (coefficient of variation: 3.3%, added 1.0 ng/ml) and 118.3% (coefficient of variation: 3.6%, added 5.0 ng/ml). The serial dilution curve for blood in bovines was parallel to that in humans. The sensitivity and the intra- and interassay coefficients of variation for the AMH assay were 0.006 ng/ml and < 8%, respectively, and for the T assay, there were 0.1 ng/ml and < 10%, respectively. The data that did not meet the threshold for the assay sensitivity were assumed to be the nadir in each assay.

Data analysis

Plasma AMH and T concentrations on Day 0 were compared among the 3 groups using the Mann-Whitney U-test. The changes between days in plasma T concentrations after the hCG stimulation test were analyzed in pairwise using the Wilcoxon signed-rank test.

Based on the function of the descended testis in the Intact and Castrated groups, the diagnostic accuracy and optimum cut-off points for plasma T concentrations were determined using the ROC curve, which was an approach validated in previous reports [24, 25]. In the present study, the sensitivity was expressed as a true-positive likelihood that calves in the Intact group had a functional testis, whereas calves in the Castrated group were used to determine specificity. The ROC curve facilitated further investigation of this phenomenon. The sensitivity designated by the various concentrations (the tentative cut-off point) on Day 0 for each hormone was plotted on the vertical axis, and [1 – each specificity] was plotted on the horizontal axis. The area under the curve (AUC) in the described ROC curve was considered to be the diagnostic accuracy of each hormone; the accuracy was higher if the derived value was proximal to 1. The optimum cut-off point was set so that the sensitivity × specificity were maximal at the tentative cut-off point, which was defined every 0.05 ng/ml. Based on the AMH levels or the optimum cut-off point for T, the sensitivity and specificity of each evaluation (AMH or T on Day 0 and T on Days 5 or 7 after the hCG stimulation test) were compared in the Intact and Castrated groups. These statistical analyses were performed using PASW® Statistics 18 (SPSS, Tokyo, Japan).

The detection rate was investigated using the AMH levels for 9 calves or the optimum cut-off point for T on Day 0 and T on
Days 5 or 7 after the hCG stimulation test for 7 calves in the Uni-crypto group.

Results

Plasma AMH concentrations before the hCG stimulation test

The plasma AMH concentrations in the Castrated group were less than 0.006 ng/ml and could not be detected by the assay due to a lack of sensitivity, so these levels were defined as 0.006 ng/ml. Plasma AMH concentrations on Day 0 were significantly higher in the Intact and Uni-crypto groups (21.1 ± 5.1 and 29.0 ± 7.5 ng/ml, respectively, Table 1 and Fig. 2) as compared with the Castrated group (P<0.01). There were no significant differences between the Intact and Uni-crypto groups (P=0.31). Therefore, based on the results in the Intact and Castrated groups, evaluating the existence of a functional testis with AMH was based on whether the plasma AMH levels in the calves were detectable or not, that is, 0.006 ng/ml or more or less than 0.006 ng/ml.

Plasma T concentrations before and after the hCG stimulation test

Plasma T concentrations on Day 0 were significantly higher in the Intact group (8.2 ± 1.7 ng/ml) as compared with 0.2 ± 0.1 ng/ml in the Castrated group and 2.4 ±1.1 ng/ml in the Uni-crypto group (P<0.01, Table 1 and Fig. 2). The plasma T concentrations on Day 0 in the Uni-crypto group were significantly higher than in the Castrated group (P<0.01). In the Intact group, the plasma T concentrations increased significantly after the hCG stimulation test to 26.8 ± 4.2 ng/ml on Day 5 compared with on Day 0, and the values then decreased significantly to 15.7 ± 3.0 ng/ml on Day 7 but were still higher than on Day 0 (P<0.01, Table 1). However, the plasma T concentrations in the Castrated group remained lower on Day 5 through Day 7. In the Uni-crypto group, the plasma T concentrations slightly increased to 3.3 ± 1.8 ng/ml on Day 5 and 3.1 ± 1.4 ng/ml on Day 7 compared with on Day 0 (P=0.56 and P=0.90 on Days 5 and 7, respectively).

Evaluation of cryptorchidism based on the detectability of AMH or the optimum cut-off point for T

The ROC curves in 36 calves (19 in the Intact group and 17 in the Castrated group) yielded AUC values of 0.99 for T. The asymptotic significance of the value was less than 0.01 in the evaluation by T levels to detect testes that descended into the scrotum. The optimum cut-off point was 0.9 ng/ml for T. The sensitivity (in the Intact group) / specificity (in the Castrated group) for detection of testes that descended into the scrotum based on the appropriate optimum cut-off point were 0.96 / 1.0 for T on Day 0 and 1.0 / 1.0 for T on Days 5 or 7 (Table 2). The detection rate in the Uni-crypto group determined using AMH detectability or the optimum cut-off point for T was 1.0 on Day 0 for the plasma AMH concentrations and 0.57 on Day 0 and 0.57 on Days 5 or 7 for the plasma T concentrations (Table 2).

Discussion

Unilateral cryptorchidism in bovine appears to be more common than bilateral cryptorchidism. The intra-abdominally retained testis is more likely to be on the left side [4, 26]. In the present study, left-side cryptorchidism was present in 5 calves, while right-side cryptorchidism was present in 4 calves.

The plasma AMH concentrations in normal male calves were about sevenfold higher than in females at birth and continued to increase until the calves were 5 months old [27]. In the present study, the plasma AMH concentrations in the Intact group at 6 months old were 1.9 ng/ml or more. The AMH levels in all calves in the Castrated group were below the threshold for assay sensitivity (<0.006 ng/ml). These findings are in agreement with previous observations of restricted expression of AMH by Sertoli cells in males [19]; moreover, the levels in blood decreased rapidly after castration [28].

The plasma T concentrations in the present intact male calves, at 6 months of age, were greater than 1.0 ng/ml, except for the concentration in one calf (0.6 ng/ml), which was similar to that in normal bulls described in previous reports [27, 29]. In general, plasma T concentrations decrease immediately after castration [29]. The plasma T concentrations in bulls castrated in the neonatal period were below 0.4 ng/ml from birth to 26 weeks of age in a previous study [30]. In the present study, the castrated calves exhibited plasma T concentrations of <0.9 ng/ml at 14 days after castration.

An intravenous injection of hCG (750 IU) stimulated T secretion in 8- to 11-month-old calves; however, the magnitude of the response in 8- to 11-month-old calves differed among the individuals [17]. In the present study, plasma T concentrations were significantly increased in 6-month-old calves in response to injection of hCG 3,000 IU. The increase in plasma T concentrations persisted for at least 7 days after the hCG stimulation test. It has been suggested that hCG might stimulate the release of T (which accumulates in Leydig cells) and increase microsomal production of enzymes involved in the synthesis of T [16].

Plasma AMH profiles have been used to evaluate the existence and function of a cryptorchid testis in cryptorchid children [31–33]. However, in the context of bovine medicine, the present study is likely the first report concerning cryptorchid calves to
use AMH profiles. In the Uni-crypto group in the present study, all levels were 0.2 ng/ml or more, that is detectable levels (more than 0.006 ng/ml). The plasma T concentrations on Day 0 in the Uni-crypto group (2.4 ± 1.1 ng/ml) were significantly higher than in the Castrated group (0.2 ± 0.1 ng/ml), while each level varied, with the levels in 3 cryptorchid calves being less than 0.9 ng/ml. Moreover, the plasma T profiles after the hCG stimulation test varied and did not show significant changes. This is in agreement with previous studies in cryptorchid bulls [34] and rats [35]. The reason for this might be abnormal function of Leydig cells in the cryptorchid testis. Leydig cells in a cryptorchid testis are somewhat hyperplasic [36]; nevertheless, only 34% of LH/hCG binding sites remained in Leydig cells of a cryptorchid testis as compared with a testis that had descended into the scrotum [37].

In the present study, based on AMH detectability and ROC curve analyses for T in the Intact and Castrated groups, both the sensitivity of AMH analysis (0.006 ng/ml) and optimum cut-off point found T (0.9 ng/ml) had higher sensitivity and specificity with respect to the existence and function of testes descended into the scrotum. When a cryptorchid testis (in the Uni-crypto group) was detected using the AMH detectability or the optimum cut-off point for T, AMH (detection rate, 1.0) was superior to T levels either before or after the hCG stimulation test (before, 0.57; after, 0.57). The hCG stimulation test in prepubertal children yielded undefined responses, which differed depending on the performance of multiple blood draws or risk for torsion after hCG administration [19]. In humans, AMH is highly sensitive for identifying undescended testicular tissue in children [31, 32]; these results are in agreement with the present study. It was concluded that one blood sample for an AMH profile is more efficient, sensitive and less time consuming than a plasma T profile before and after an hCG stimulation test for evaluating the existence of a functional cryptorchid testis in Japanese Black calves. Moreover, the AMH profile could be used as a confirmatory marker in supposedly castrated animals. Further studies might be needed to clarify the regulation of AMH secreted by Sertoli cells in the normal and cryptorchid testis and improve the precision of these findings.

Table 1. Profiles of AMH and T after the hCG stimulation test in the respective calf groups

<table>
<thead>
<tr>
<th>Group¹(n)</th>
<th>AMH</th>
<th>T after the hCG stimulation test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 0²</td>
</tr>
<tr>
<td>Intact (n=19)</td>
<td>Mean ± SEM</td>
<td>21.1 ± 5.1A</td>
</tr>
<tr>
<td></td>
<td>Min.</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>Max.</td>
<td>68.7</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>9.9</td>
</tr>
<tr>
<td>Castrated (n=17)</td>
<td>Mean ± SEM</td>
<td>0.006B</td>
</tr>
<tr>
<td></td>
<td>Min.</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>Max.</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>0.006</td>
</tr>
<tr>
<td>Uni-crypto (n=9 in AMH, 7 in T³)</td>
<td>Mean ± SEM</td>
<td>29.0 ± 7.5A</td>
</tr>
<tr>
<td></td>
<td>Min.</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>Max.</td>
<td>64.5</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>26.8</td>
</tr>
</tbody>
</table>

¹) Intact: intact male calves. Castrated: bilaterally castrated calves. Uni-crypto: unilateral cryptorchid calves after castration of the descended testis. ²) Day 0: before hCG stimulation test. Days 5 and 7: after hCG stimulation test. ³) In the Uni-crypto group, AMH levels were evaluated in 9 calves; T levels were evaluated in 7 calves. Mean ± SEM values that differ significantly are indicated by capital letters (between the different groups) or small letters (between different days in the same group). A–C, e–f P<0.05. Different letters indicate P<0.01.
In AMH, 0.006 ng/ml was the sensitivity of the AMH assay, and it reflected whether AMH was detectable or not. The optimum cut-off point for T was determined based on the observed sensitivity × specificity, which became the values for the existence and function of a descended testis, at various concentrations. 1) Sensitivity in evaluation: the ratio that was higher than the sensitivity of the AMH assay or the optimum cut-off point for T in each calf in the Intact group (n=19). Specificity in evaluation: the ratio that was less than the sensitivity of the AMH assay or the optimum cut-off point for T in each calf in the Castrated group (n=17). 2) Detection rate: the ratio that was higher than the sensitivity of the AMH assay or the optimum cut-off point for T in each calf in the Uni-crypto group. * In the Uni-crypto group, AMH levels were evaluated in 9 calves. ** In the Uni-crypto group, T levels were evaluated in 7 calves.

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AMH PROFILE TO EVALUATE CRYPTORCHID TESTIS


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