

Biological and clinical significance of anti-Müllerian hormone determination in blood serum of the mare

J. Almeida^a, B.A. Ball^{a,*}, A.J. Conley^a, N.J. Place^b, I.K.M. Liu^a, E.L. Scholtz^a,
L. Mathewson^a, S.D. Stanley^c, B.C. Moeller^c

^a Department of Population Health and Reproduction, California Animal Health and Food Safety Laboratory, School of Veterinary Medicine, University of California, Davis, California, USA

^b Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York, USA

^c K.L. Maddy Equine Analytical Chemistry Laboratory, School of Veterinary Medicine, University of California, Davis, California, USA

Received 9 March 2011; received in revised form 26 May 2011; accepted 4 June 2011

Abstract

Anti-Müllerian hormone (AMH), a member of the transforming growth factor β superfamily of growth and differentiation factors, is expressed in granulosa cells of preantral and small antral ovarian follicles. In humans, AMH appeared to regulate recruitment and growth of small ovarian follicles. Furthermore, circulating AMH concentrations were elevated in women with granulosa-cell tumors (GCT). In the horse, GCTs are the most common tumor of the ovary, and a variety of endocrine assays have been used to diagnose presumptive GCTs. The objectives of the present study were to validate a heterologous enzyme immunoassay for determination of serum AMH in the horse, and to determine concentrations of AMH in the blood of mares during the estrous cycle, pregnancy, and in mares with granulosa-cell tumors. Mares with normal estrous cycles ($n = 6$) and pregnant mares ($n = 6$) had blood samples collected throughout one interovulatory period and monthly throughout gestation, respectively. Mares diagnosed with GCT had blood samples taken before ($n = 11$) and after ovariectomy ($n = 5$). Tumors were sectioned and fixed for immunohistochemistry and snap frozen for immunoblot analyses and RT-qPCR. In normal cyclic mares and in pregnant mares, there was no effect of cycle stage or month of gestation on serum AMH concentrations. In GCT mares, serum concentrations of AMH (1901.4 ± 1144.6 ng/mL) were higher than those in cyclic (0.96 ± 0.08 ng/mL) or pregnant (0.72 ± 0.05 ng/mL) mares and decreased after tumor removal. Both AMH and AMH receptor (AMHR2) immunolabeling and expression were detected by immunohistochemistry in the tumor and cyst fluid obtained from mares with GCTs. Therefore, we concluded that AMH was a useful biomarker for detection of granulosa-cell tumors in mares.

© 2011 Elsevier Inc. All rights reserved.

Keywords: Anti-Müllerian hormone; Granulosa-cell tumor; Ovary; Ovarian follicle; Pregnancy; Equine estrous cycle

1. Introduction

Anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance (MIS), is a 140-kD dimeric glycoprotein and a member of the transforming

growth factor β superfamily of growth and differentiation factors [1]. During sexual differentiation in males, testosterone is responsible for Wolffian (mesonephric) duct development, whereas AMH is required for Müllerian (paramesonephric) duct regression [2]. In females, AMH is not expressed during the period of sexual differentiation, assuring normal development of the female genital tract [3,4]. However, after birth,

* Corresponding author. Tel.: 859-218-1141; fax: 859-257-8542.
E-mail address: b.a.ball@uky.edu (B. Ball).

AMH is expressed in granulosa cells of preantral and small antral follicles in the ovary, and AMH inhibits recruitment of primordial follicles into the pool of growing follicles and decreases responsiveness of growing follicles to FSH [5]. In the rat, AMH is involved in the control of follicular development [6], based upon the patterns of expression of AMH and AMH receptor type II in the postnatal ovary [7]. In women, AMH expression begins in primary follicles, increases gradually and reaches its peak in preantral and antral follicles at approximately 4 mm in diameter. After this threshold, the expression of AMH decreases, becoming undetectable when the follicles reach a diameter of 8 mm [8]. This pattern of expression was consistent with the hypothesis that AMH inhibits the recruitment of primordial follicles and reduces the stimulatory effect of FSH on the growth of preantral and small antral follicles.

Although several studies reported serum AMH concentration during the menstrual cycle and pregnancy in women [9–14], it is not clear whether there is a significant change in AMH concentration during the menstrual cycle or pregnancy. Serum AMH concentrations have not been previously reported in the mare; however, our laboratory has characterized AMH expression in normal equine ovaries and in ovaries from mares with granulosa-cell tumors [15]. In normal equine ovaries, AMH expression was greatest in small growing follicles and in small antral follicles and expression of AMH was reduced in larger antral follicles. In equine granulosa-cell tumors, AMH was strongly but variably expressed in the granulosa-like cells, based upon immunohistochemistry [15].

Determination of circulating AMH concentrations has been used to monitor granulosa-cell tumors (GCT) in women [16]. A study conducted by La Marca and Volpe [17], demonstrated elevated AMH levels in 76–93% of women with GCTs and normalization of AMH concentrations after tumor removal. Furthermore, Rey et al [18] demonstrated positive immunostaining for AMH in GCTs from women. Therefore, determination of serum AMH concentrations has become an increasingly useful assay for evaluation of a number of reproductive disorders in humans.

In the horse, GCTs are the most common tumor of the ovary and account for 85% of equine reproductive tract neoplasms [19] and 2.5% of all neoplasms in horses [20]. According to MacLachlan [21], GCTs are composed of multiple layers of granulosa-like cells within the follicular structure and a supporting stroma that may contain theca-like cells [22]. Even though

malignant GCTs have been reported [23], most GCTs in the mare are benign. A variety of endocrine assays have been used to diagnose presumptive GCTs in the horse including inhibin, testosterone and progesterone [24]. We hypothesized that AMH might be a useful biomarker for GCTs in the mare, and that the development of a sensitive immunoassay for equine AMH might have an application in detection of equine GCTs.

The objectives of the study reported here were to: (i) validate a heterologous enzyme immunoassay for determination of serum anti-Müllerian hormone in the horse; (ii) determine concentrations of AMH in the blood of mares during the estrous cycle and pregnancy, as well as to determine serum AMH concentrations in mares with granulosa-cell tumors; and (iii) characterize AMH and AMH receptor (AMHR2) localization and mRNA expression in the normal ovary and in granulosa-cell tumors from mares.

2. Materials and methods

2.1. Blood sample collection from mares with normal estrous cycles, pregnant mares and ovariectomized mares

Mares with normal estrous cycles ($n = 6$) from the Center of Equine Health at the University of California, Davis had blood serum and plasma collected every Monday, Wednesday and Friday throughout one inter-ovulatory period. Pregnant mares ($n = 6$) from UC-Davis Animal Science Horse Barn had blood serum collected monthly throughout gestation for determination of AMH concentration. Ovariectomized mares ($n = 5$) from the Center of Equine Health at University of California, Davis had blood serum collected once. The blood was centrifuged, and the sera were stored at $-20\text{ }^{\circ}\text{C}$ until assayed.

2.2. Blood and tissue collection from mares with granulosa-cell tumors

Mares ($n = 11$) with confirmed GCTs that were presented to the Veterinary Medical Teaching Hospital at the University of California, Davis or to private veterinary clinics, had blood serum collected one time or on multiple occasions ($n = 5$ mares) prior to removal of the ovarian tumor. Five of eleven mares also had serum collected after ovariectomy. After surgical removal, tumors ($n = 11$) were fixed in formalin and sectioned for histopathology and immunohistochemistry. Tissue from GCTs was also snap frozen on dry ice for immunoblot analyses and RT-qPCR. Expression of

AMH in GCT tissue and cyst fluid was analyzed by immunoblotting, and tumor cyst fluid was analyzed by ELISA to determine AMH concentrations. In all mares, serum samples were assayed for determination of circulating concentrations of inhibin, testosterone and progesterone.

2.3. Serum and cyst fluid analyses

Serum and cyst fluid AMH concentrations were determined with an enzyme immunoassay (Active AMH-ELISA #DSL-10-14400; Diagnostic Systems Laboratories, Webster, TX, USA), according to the manufacturer's directions. Briefly, 20 μ L of the standards containing various concentrations of AMH, controls, and samples were incubated in an anti-AMH antibody coated microtitration plate. To keep assay results within the standard curve, some samples (with high AMH) concentrations were diluted at 1:100 or 1:1,000 in standard buffer. After incubation and washing, anti-AMH biotin conjugate was added to each well. After a second incubation and washing step, streptavidin-horseradish peroxidase (HRP) was added to each well. After a third incubation and washing step, the substrate, tetramethylbenzidine (TMB), was added to the wells, followed by addition of an acidic stop solution. The degree of enzymatic turnover of the substrate was determined by dual wavelength absorbance measurement at 450 and 598 nm in a microplate spectrophotometer (BioTek Powerwave HT, Winooski, VT, USA). In order to test parallelism of the assay, serially diluted blood serum from two mares with GCT and one normal cyclic mare were assayed and compared to the standard curve included in the assay kit.

Serum concentrations of inhibin, testosterone and progesterone in sera from mares with GCT were determined in the Clinical Endocrinology Laboratory at the University of California, Davis using immunoassays as validated and described by Roser et al [25], Stabenfeldt et al [26], and Munro and Stabenfeldt [27], respectively.

Plasma progesterone concentrations from cycling mares were determined by liquid chromatography-mass spectrometry (LC-MS) at the California Animal Health and Food Safety Laboratory System (CAHFS) at UC Davis. Briefly, concentrations of progesterone were measured by using a Cohesive Aria TLX2 Turbulent Flow liquid chromatography system coupled to a Thermo Quantum Ultra triple quadrupole mass spectrometer with an atmospheric pressure chemical ionization source operating in the positive ion mode. Deuterated-testosterone was used as an internal standard for

each sample and similarly processed gelding plasma was run for quality control and calibration curve samples. Plasma was diluted with water containing the internal standard and progesterone extracted using a Thermo Cyclone turbulent flow column. Following isolation of analytes on the first column, progesterone and the internal standard were separated using an ACE C18 column with a linear gradient of acetonitrile and water both with 0.2% formic acid at a flow rate of 0.35 mL/min. Detection and quantification were accomplished using selective reaction monitoring 5 MS/MS product ion transitions for progesterone (mass to charge ratio (m/z) 315.2 \rightarrow 79.1, 81.0, 91.0, 97.1, and 109.1), with the m/z 97.1 transition used for quantification. The concentration of progesterone in each sample was determined in duplicate by the internal standard method using the peak area ratio and linear regression analysis. Progesterone had a lower limit of quantification of 0.5 ng/mL in equine plasma.

2.4. Immunohistochemistry

Fixed tissues were deparaffinized through CitriSolv (Fisher Scientific, Pittsburg, PA, USA), dehydrated through a graded alcohol series (100, 95, and 70% ethanol), and endogenase peroxidases were quenched with 0.3% H₂O₂ in methanol for 30 min. Antigen retrieval was performed with antigen unmasking solution (Vectorlabs; Burlingame, CA, USA) for 5 min at 93 °C by steaming. After rinsing in PBS, slides were blocked with either normal rabbit sera (for slides to be incubated with AMHR2) or normal goat sera (for slides to be incubated with AMH primary antibody) for 20 min at 20 °C. Sections were then incubated individually with the goat polyclonal antisera (1:500) directed against a C-terminal peptide antigen based upon human AMH (sc-6886; Santa Cruz Biotechnology; Santa Cruz, CA, USA; [15]) and rabbit polyclonal antisera (1:200) directed against N-terminal region of human AMHR2 (AP7111a; ABGENT; San Diego, CA, USA) in humidified chambers overnight at 4 °C.

After incubation with the primary antisera, slides were rinsed for 5 min in PBS and incubated with a biotinylated second antisera (1:2000; donkey anti-goat IgG or goat anti-rabbit IgG) for 30 min prior to detection using the Vectastain ABC detection kit for slides incubated with AMH and the Vector NovaRed detection kit (Vectorlabs; Burlingame, CA, USA) for slides incubated with AMHR2 primary antisera.

Slides incubated with AMH were counterstained with hematoxylin and mounted in aqueous mounting media (DakoCytomation, Carpinteria, CA, USA). Slides incu-

bated with AMHR2 primary antisera were counterstained with hematoxylin and submitted to rehydration through a graded alcohol series prior to mounting with Permount.

To determine specificity of primary antisera labeling, slides were incubated with normal rabbit or goat sera, as appropriate. As a further control, AMH and AMHR2 were preadsorbed with their respective blocking peptide at 5/1 (w/w) overnight at 4 °C prior to use in immunolabeling studies.

2.5. Immunoblot analysis

Approximately 50 µg of GCT tissue and 20 µL of cyst fluid from a granulosa-cell tumor, were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto polyvinylidene fluoride (PVDF) membranes (Immobilon P, Millipore Corp., Bedford, MA, USA) under reducing conditions. Then, AMH was detected with a polyclonal antibodies produced in our laboratory against a peptide antigen derived from the equine AMH sequence: antisera # 0916 (1952.15 Da; aa117 → Acetyl-CSPADRQAAVPSLQRLQA-amide). Immunoreactive proteins were detected with HRP-linked IgG (1:10,000) and luminol reagents with electrochemiluminescence (Amersham, Arlington Heights, IL, USA).

2.6. Quantitative real time PCR

Ribonucleic acid (RNA) was extracted from a small amount of previously snap frozen tissue (< 30 mg) using Qiagen's RNeasy Mini Kit. Generation of cDNA was done with Qiagen's QuantiTect Reverse Transcription Kit, which had an integrated genomic DNA removal step. Primers specific for the known sequence of AMH and AMHR2 (Table 1) were designed using Roche's Universal Probe primer design website (<https://www.roche-applied-science.com>). Equine β2-microglobulin sequence was obtained from UCSC horse genome and used as reference gene [28]. For PCR, the resulting amplicons were between 80–120 bps. Primers were used at 100 µM concentration and probes (8 bp

with a fluorescent (6-carboxy-fluorescein) label) were used at 10 µM. Qiagen's QuantiTect Probe PCR kit contained a HotStarTaq DNA polymerase, ROX passive reference dye, and a dNTP mix in an optimized buffer. The cycling conditions for qPCR were an initial incubation at 95 °C for 15 min, followed by 95 °C for 15 s, and 60 °C for 60 s, repeated 40 times. Amplification was measured using an ABI 7900, available through the Lucy Whittier Molecular and Diagnostic Core. Samples were extracted from 9 GCT mares and 11 normal ovaries (contralateral ovaries from GCT mares, n = 3; and normal ovaries from routine ovariectomies at the Veterinary Medical Teaching Hospital at the University of California, n = 8) for AMH analyses and eight GCT and 11 normal for AMHR2.

2.7. Statistical analyses

For RT-qPCR analyses, the difference between threshold cycles (ΔCt) was calculated by subtracting the Ct value of target gene from that of reference gene, and subjected to ANOVA to generate ΔΔCt values ([29]; JMP ver 8; SAS, Cary, NC, USA). Repeated measure ANOVA was used to examine AMH concentration from normal cyclic mares as well as from pregnant mares (JMP ver 8). Because of the small sample size, statistical comparisons were not performed between mares with GCTs and normal cyclic or pregnant mares. Relationships between serum concentrations of endocrine markers in mares with GCT were examined using Spearman's rho (JMP ver 8). Data were expressed as mean ± SEM.

3. Results

3.1. Validation

To test the parallelism of the heterologous AMH assay, blood serum from two mares diagnosed with granulosa-cell tumors and one normal cyclic mare were serially diluted in a protein based standard buffer and assayed

Table 1

PCR primer sequence and respective efficiencies for anti-Müllerian hormone (AMH), anti-Müllerian hormone receptor II (AMHR2) and equine β2-microglobulin (Eqβ2M) genes used for real-time quantitative PCR.

Gene name	Reference number	Primers	Primer efficiency (%)
AMH	GenBank accession # JF330269.1	Forward: 5' – ccatctggaggagccaac – 3' Reverse: 3' – ccegtgacagtgcctcag – 5'	97.3
AMHR2	GenBank accession # AYO32721.1	Forward: 5' – ctagacttgcgcctgaca – 3' Reverse: 3' – gccagctctgcctcata – 5'	100
Eqβ2M	Equine Genome accession # NM_001082502.	Forward: 5' – gatagttaaagtggatcgagacctct – 3' Reverse: 3' – gcacaatttgaactagctcaatcc – 5'	100

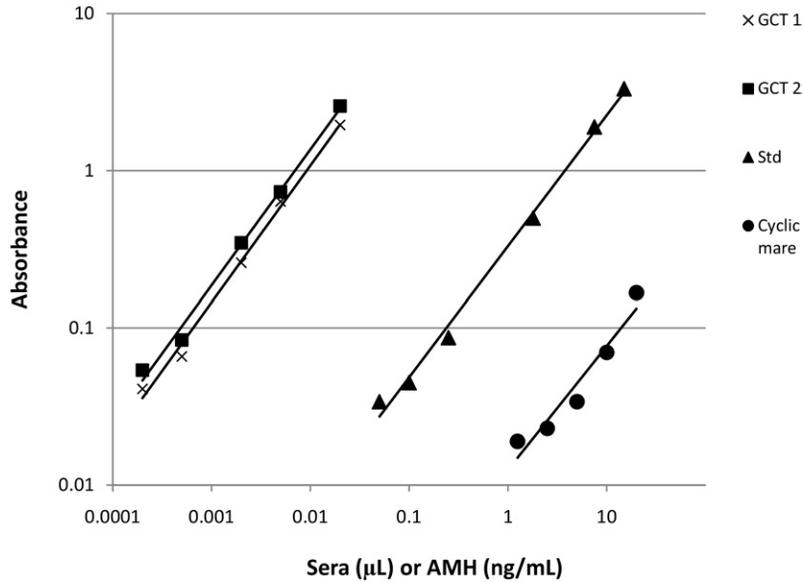


Fig. 1. Parallelism plot for serum from two mares with granulosa-cell tumors and one normal cyclic mare compared with assay standards for the DSL anti-Müllerian hormone (AMH) assay.

along with standards. Dilution curves were parallel to the standard curve within the standard range (Fig. 1).

3.2. Intra-assay and inter-assay coefficients of variation

The intra-assay and inter-assay coefficients of variation (CV) for the low AMH control (1.8 ng/mL) were 3.1 and 7.8% respectively. Intra-assay and interassay CVs for the high AMH control (8 ng/mL) were 1.9 and 4.7%, respectively. In addition, an equine control which consisted of serum from one mare with a confirmed GCT was run in quadruplicate in each assay (1:1000 dilution). Intra-assay and inter-assay CVs for this sample were 2.3 and 15.2%, respectively.

3.3. Normal cyclic mares and ovariectomized mares

Mean serum AMH concentration for ovariectomized mares was 0.06 ± 0.003 ng/mL (range: 0.05–0.07 ng/mL) and was not different from blank values. Serum AMH concentrations in cyclic mares and pregnant mares appeared higher than those in ovariectomized mares. In normal cyclic mares, mean serum AMH concentration was 0.96 ± 0.08 ng/mL (range: 0.22–2.94 ng/mL), and there was no effect ($P > 0.77$) of cycle stage on serum AMH concentrations (Fig. 2); however, AMH concentrations were different ($P < 0.001$) among mares. In pregnant mares, mean serum AMH concentration was 0.72 ± 0.05 ng/mL (range: 0.26–2.61 ng/mL), and there was no significant effect of month of

gestation on AMH concentrations; however, there were differences among mares ($P < 0.001$; Fig. 3). Progesterone concentrations in cyclic mares confirmed their physiological state.

3.4. Granulosa cell tumor mares

In mares ($n = 11$) with GCTs confirmed by histopathology, serum concentrations of AMH were $1,901.4 \pm 1,144.6$ ng/mL (range: 14–10,596 ng/mL). Mean concentration of AMH in cyst fluid recovered from GCTs ($n = 4$) was $6,768.6 \pm 3,857.3$ ng/mL (range: 80.3–9,482.0 ng/mL). Concentrations of AMH in sera and cyst fluid from GCT mares appeared higher than serum AMH concentrations determined in ovariectomized, cyclic or pregnant mares. Five of the 11 mares had blood drawn repeatedly before tumor removal and these samples were assayed for AMH, inhibin, testosterone, and progesterone concentrations (Table 2). In four of these five mares, serum AMH concentrations increased over sampling intervals of 31 to 297 d. In the fifth mare, serum AMH, inhibin and testosterone concentrations declined over an interval of 83 d. In five mares in which blood samples were available after removal of the granulosa-cell tumor, serum AMH concentrations declined (Table 3 and Fig. 4), and half-life of hormone disappearance from serum was estimated at 1.9 d. Eight out of these eleven mares with confirmed GCT had elevated concentrations of inhibin (> 0.7 ng/mL; Table 4) when compared to normal cycling mares, whereas three

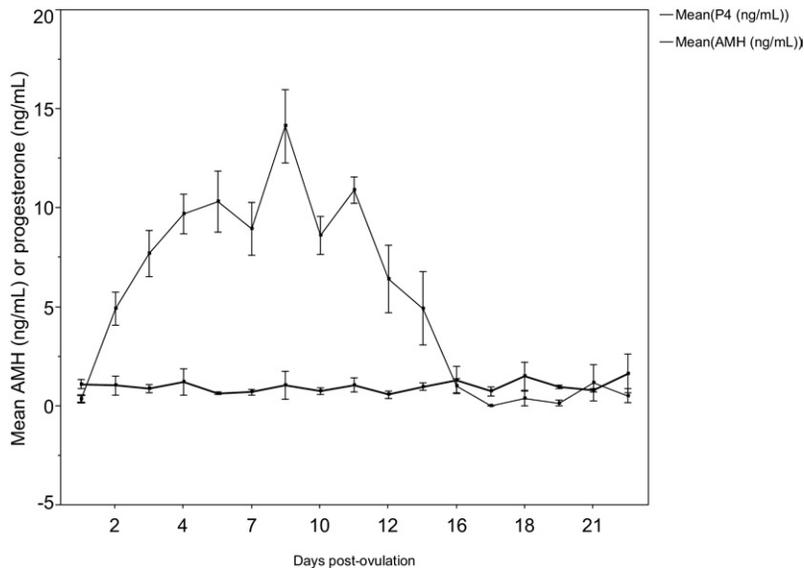


Fig. 2. Mean (\pm SEM) serum concentrations of anti-Müllerian hormone (AMH) and progesterone throughout the estrous cycle in cycling mares ($n = 6$). Samples were collected Monday, Wednesday and Friday throughout one interovulatory period.

mares with GCT had inhibin concentrations < 0.7 ng/mL. Five of 11 mares with a GCT had increased testosterone concentrations (> 45 pg/mL; Table 4). All mares with GCT had low concentrations of progesterone (< 1.0 ng/mL; Table 4), indicating a lack of luteal tissue. In mares with GCTs, serum concentrations of inhibin and AMH were positively associated (Spearman's $\rho = 0.71$; $P < 0.02$).

3.5. Immunohistochemistry analyses

Anti-Müllerian hormone (AMH) and its receptor (AMHR2) immunolabeling was observed in all equine

GCTs. Immunostaining for AMH and AMHR2 was observed in the granulosa components of granulosa cell tumors (Fig. 5a and 5b) and varied in distribution within tissue sections. Furthermore, AMH and AMHR2 immunolabeling was also observed in underlying theca-like cells in the tumors. Specificity of the immunolabeling for AMH and AMHR2 was demonstrated by the complete loss of immunostaining when the primary AMH [14] and AMHR2 antibody was pre-incubated with the corresponding peptide (Fig. 5c), or when the primary AMH or AMHR2 antibody was omitted (data not shown).

3.6. Immunoblot analyses

Immunoblotting of equine granulosa-cell tumor and tumor cyst fluid (Fig. 6) using a polyclonal antibody directed against an 18-mer peptide antigen derived from the N-terminus of equine AMH revealed major immunoreactive proteins at approximately 62 and 130 kDa, with minor immunoreactive proteins at 90 and 45 kDa.

3.7. Real time quantitative PCR analyses

Anti-Müllerian hormone (AMH) mRNA expression was higher in granulosa-cell tumors when compared to normal ovary (26.7 fold increase in expression; $P = 0.01$). Expression of AMHR2 mRNA in granulosa-cell tumors was not different from a normal ovary (1.4 fold increase in expression; $P = 0.3$).

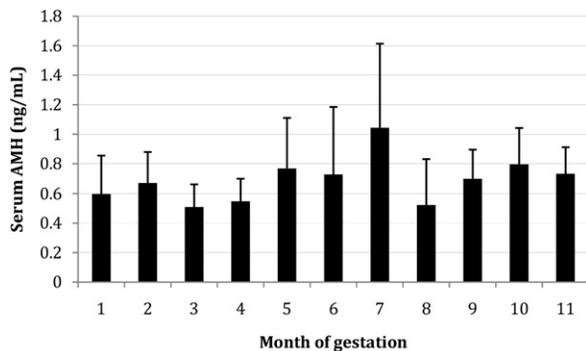


Fig. 3. Mean anti-Müllerian hormone (AMH) concentrations throughout pregnancy in the mare ($n = 6$). Samples were collected monthly. There was no effect of month of gestation on serum AMH concentrations; however, there were significant mare effects on AMH. Each error bar represents one SEM.

Table 2

Serum concentrations of anti-Müllerian hormone (AMH), inhibin, testosterone, and progesterone in mares with confirmed granulosa-cell tumors and repeated sampling prior to surgical removal of the tumor. Sampling intervals are expressed relative to the first sample (Day 0) for each mare. In four of five mares, serum AMH, inhibin and testosterone increased over sampling intervals of 31 to 297 d.

Mare #	AMH (ng/mL)	Inhibin (ng/mL)	Testosterone(pg/mL)	Progesterone(ng/mL)	Interval (d) between blood collections
1	9.0	0.71	34.6	0.2	0
	11.1	0.56	17.7	0.1	53
	14.1	0.44	23.6	0.1	82
2	245.0	2.08	36.4	0.2	0
	414.0	8.93	42.9	0.1	297
3	89.6	1.32	88.5	0.1	0
	664.2	1.93	57.8	0.3	83
4	83.8	3.26	68.8	0.1	0
	54.3	2.50	99.6	0.4	84
5	63.5	0.98	24.1	0.1	0
	82.9	1.33	29.7	0.2	31

4. Discussion

In the present study, we report physiological concentrations of circulating AMH throughout the estrous cycle and pregnancy in mares. Ovariectomized mares had AMH concentrations at or below the limit of detection of the assay, confirming the ovarian source of AMH in mares. Concentrations of AMH were higher in cyclic and pregnant mares when compared to ovariectomized mares, and AMH concentrations did not change significantly throughout the estrous cycle or pregnancy. In several studies, serum AMH concentrations were measured at various times during the menstrual cycle of women; results differed among studies regarding the effect of cycle stage on AMH concentrations. Two studies in women [9,30] found higher AMH concentrations during the follicular phase, but

several other studies did not detect significant changes in AMH concentration throughout the menstrual cycle [11,31,32]. It is known that AMH is expressed by early primary follicles up through the antral stages of FSH-dependent growth, which suggests an inhibitory role for AMH at two distinct stages of folliculogenesis: one during the transition of follicles from primordial into maturational stages and another during follicular sensitivity to FSH. Since the recruitable pool represents a constantly cycling population of follicles, peripherally detectable AMH may remain relatively stable within cycles and, possibly, between cycles. In the present study, there was no significant variation in AMH concentration by day of the estrous cycle in

Table 3

Change in serum anti-Müllerian hormone (AMH) concentrations before and after surgical removal of the tumor in mares diagnosed with granulosa-cell tumors.

Mare #	Time of blood collection (relative to ovariectomy)	AMH final (ng/mL)	Interval
1	Before	10,596.0	1 d
	After	5,459.0	
2	Before	90.2	2 d
	After	42.0	
3	Before	664.2	2 wk
	After	211.6	
4	Before	49.4	2 d
	After	33.2	
5	Before	74.2	1 d
	After	55.2	
	After	32.5	
	After	25.7	3 d

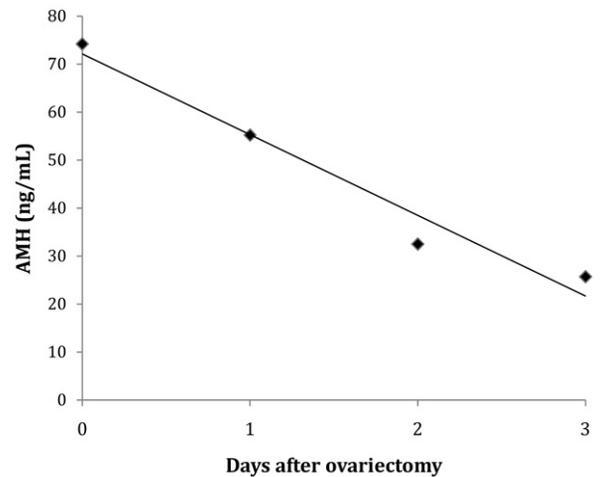


Fig. 4. Decline in serum anti-Müllerian hormone (AMH) concentrations after surgical removal of a granulosa-cell tumor in Mare #5 from Table 3.

Table 4

Serum concentrations of anti-Müllerian hormone (AMH), inhibin, testosterone, and progesterone in mares with confirmed granulosa-cell tumors and respective tumor masses.

Mare #	AMH(ng/mL)	Inhibin(ng/mL)	Testosterone(pg/mL)	Progesterone(ng/mL)	Estimated tumor mass (g)
1	10,596.0	10.0	45.2	0.2	8000
2	229.0	1.39	183.6	1.0	—
3	664.2	1.93	57.8	0.3	113
4	49.4	0.00	41.4	0.1	—
5	74.2	2.01	191.7	0.1	—
6	414.0	8.93	42.9	0.1	588
7	14.1	0.44	23.6	0.1	296
8	8,416.0	10.0	97.0	0.3	8500
9	321.7	0.54	43.6	0.1	270
10	54.3	2.50	99.6	0.4	7235
11	82.9	1.33	29.7	0.2	288

mares, which may be due to a variable population of small follicles.

In the present study, AMH concentrations differed among mares. In women, two patterns of changes in circulating AMH have been described [10], and differences in circulating AMH concentration appeared to be related to the population of oocytes remaining in the ovary [33,34]. The “aging ovary” pattern was characterized by low AMH concentrations with little variation throughout menstrual cycle; this pattern appeared to be associated with a decreased ovarian reserve. The “younger ovary” pattern included higher AMH concentrations with significant variation between Days 2 to 7 of menstrual cycle, which coincides with the follicular phase in women. It is believed that the source of AMH that enters the blood circulation represent the cohort of ultrasonically visible antral follicles, through the phase at which AMH expression disappears in follicles > 7 mm in diameter [33]. In the present study, the mares examined were between 6 to 13 y of age. Even though the sample size was not large, differences in circulating AMH concentrations may reflect differences among ovarian reserves in each mare. Further studies should be conducted in order to correlate AMH concentration and the number of primordial and antral follicles in the mare.

Serum AMH concentrations in pregnant mares were similar to concentrations during the estrous cycle. Our findings in cyclic and pregnant mares were in agreement with studies conducted in women by La Marca et al [12] and Nelson et al [35], in which differences in circulating AMH concentrations between pregnancy and menstrual cycle were not observed.

To evaluate circulating AMH as a biomarker for GCTs in mares, we used the enzyme-linked immunosorbent assay (ELISA) to examine serum concentra-

tions of AMH in mares which had GCTs confirmed by histopathology. All mares diagnosed with GCTs had increased serum AMH concentrations when compared to ovariectomized mares, normal cyclic mares, or pregnant mares. In addition to increased serum AMH concentrations, cyst fluid obtained from GCTs also had elevated AMH concentrations, supporting the increased local production of AMH, as would be expected. After surgical removal of GCTs, AMH concentrations decreased approximately 50% within 48 h after tumor removal; therefore, we inferred that the increased AMH concentration detected in mares with GCTs was derived from the ovarian tumor. Based upon the decline in AMH after removal of GCTs in mares, it appeared that half-life of AMH in serum in the mare was approximately 2 d, similar to the half-life reported for AMH in cattle [36].

Serum AMH concentrations have also been used in women for detection of GCTs. Gustafson et al [37] detected elevated serum concentrations of AMH in four women with GCTs, and serum AMH concentrations were normal in six women who had previously undergone resection of GCTs. Elevations in serum AMH appeared to be specific to GCTs, since AMH concentrations were not elevated in other types of ovarian or gynecologic neoplasia. Rey et al [38] found normal AMH concentrations in 93.3% of patients with non-gynecologic cancer or gynecologic cancer other than GCTs. Conversely, AMH concentrations were above the normal range in eight of nine patients with progressive GCTs, whereas AMH was below the detection limit in one patient with clinical remission of the disease. According to Rey et al [38], 11 patients with recurrent GCT had a good correlation of AMH with evolution of the disease during follow-up in nine cases. Serum AMH fell to undetectable values after successful

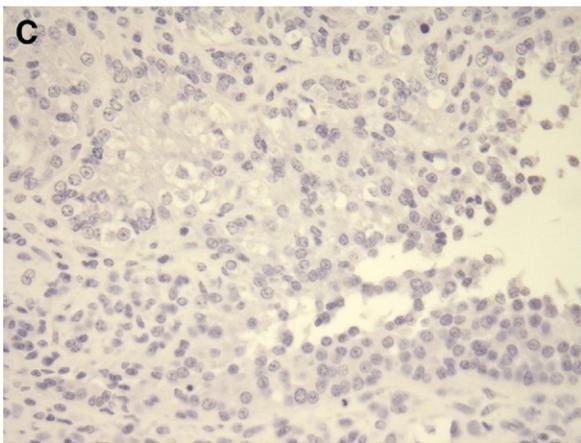
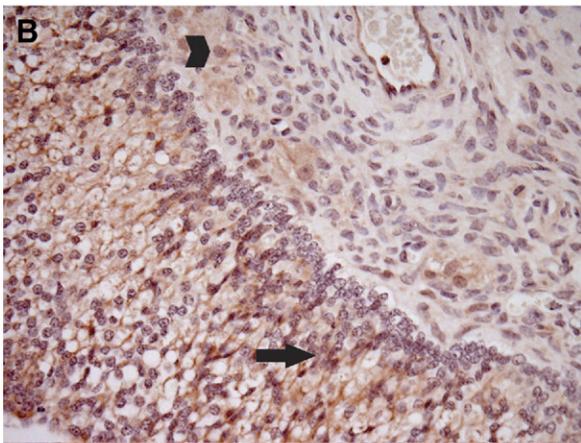
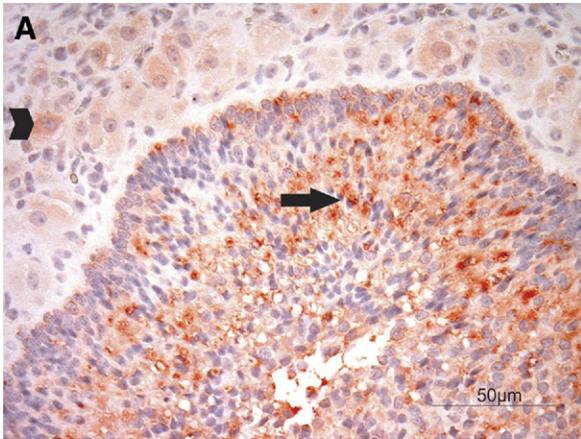


Fig. 5. Photomicrographs of equine tissue demonstrating immunoreactive anti-Müllerian hormone (AMH) (A) and AMHR2 (B). AMH (A) and AMHR2 (B) immunolabeling was observed in the granulosa components (arrow) of granulosa cell tumors and showed heterogeneity within a tissue section and among various tissues. AMH (A) and AMHR2 (B) immunolabeling was also observed in underlying theca-like cells (arrowhead) in the tumors. Pre-incubation of the primary antibody with the corresponding blocking peptide completely eliminated AMH (previous study, [16]) and AMHR2 immunolabeling (C).

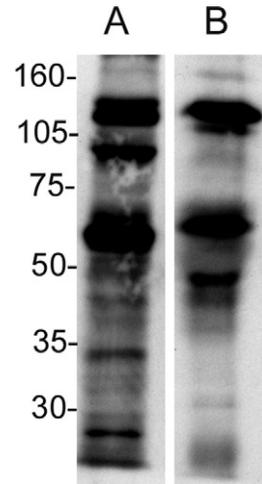


Fig. 6. Immunoblot analysis of anti-Müllerian hormone (AMH) in GCT tissue (A) and cyst fluid (B) from mare diagnosed with a granulosa cell tumor. Immunoblot was probed with antisera # 0916. Proteins were separated in polyacrylamide gel electrophoresis (12%) under reducing conditions.

treatment or rose early in cases of recurrence of the disease. Therefore, AMH may be used to evaluate the efficacy of the treatment. Based upon the present study, serum AMH concentrations appeared to be a sensitive and specific marker for granulosa-cell tumor diagnosis in the mare.

In the present study, increased AMH concentrations were detected in all the mares with confirmed GCTs, whereas increased concentrations of inhibin or testosterone were detected in 73% or 45% of these mares, respectively. The proportion of mares with changes in inhibin or testosterone appeared similar to those of earlier studies which found elevated inhibin (87%) or testosterone (40 to 50%) concentrations in mares with GCTs [24]. Serum concentrations of AMH and inhibin were positively correlated in mares with GCTs, similar to findings in women [38]. This association between serum AMH and inhibin concentrations in mares with GCTs likely reflected the common origin of these hormones from the granulosa-cells of these neoplasms. Based upon the limited number of mares with GCTs that were examined in the present study, AMH may represent a more specific serum parameter for GCT diagnosis when compared to testosterone, inhibin and progesterone.

In order to confirm the presence of AMH in the GCTs used in our study, immunohistochemistry and immunoblot analyses were performed. Immunoreactive AMH and AMHR2 were observed in all GCTs examined, primarily within the granulosa-like component of

the tumors. As previously reported by Ball et al [15], there was considerable heterogeneity of AMH immunostaining within tumors and among tumors from different mares. Immunoblotting of GCT cyst fluid and tissue revealed numerous proteins which likely represented the monomer and dimer of AMH, along with cleavage products of this protein. In addition to immunoreactive AMH, AMH mRNA was significantly higher in GCT tumors when compared to normal ovaries, whereas expression of AMHR2 mRNA was not different between GCTs and normal ovaries. Therefore, we inferred that immunoreactive AMH was expressed within the granulosa cell component of equine granulosa-cell tumors and that bioactive AMH can be detected in sera from mares with GCTs.

It has been postulated that the suppressed follicular activity often noted clinically in the contralateral (normal) ovary of mares with GCTs might be due to suppression of FSH associated with increased inhibin or other hormones from the GCT [24,26]. The pathophysiology of ovarian atrophy in mares with GCTs is controversial. Bailey et al [39] reported decreased FSH and basal GnRH concentrations in mares with GCTs, but Zelli et al [40] did not find significant differences in FSH and basal GnRH concentrations in mares with GCTs when compared to normal mares. Based upon our current results, we hypothesize that the increased AMH production in mares with GCTs may suppress the effects of FSH on follicular development, with subsequent atrophy of the remaining normal ovary.

In conclusion, this is apparently the first report detailing circulating concentrations of anti-Müllerian hormone (AMH) in normal cyclic and pregnant mares, as well as in those with confirmed GCTs. There were no significant changes in serum AMH concentrations during the estrous cycle or pregnancy, although there were significant mare effects on AMH concentrations in both cyclic and pregnant mares. Serum AMH concentrations were markedly elevated in mares with confirmed GCTs, and serum AMH declined after removal of the ovarian tumor from mares. Based on these data, we concluded that AMH was expressed in equine GCTs and that serum AMH concentration was a useful biomarker for the diagnosis of GCTs in the mare.

Acknowledgments

This study was supported the John P. Hughes Endowment, UC Davis Center for Equine Health, CAPES, and Fulbright.

References

- [1] Cate RL, Mattaliano RJ, Hession C, Tizard R, Farber NM, Cheung A, Ninfa EG, Frey AZ, Gash DJ, Chow EP, et al. Isolation of the bovine and human genes for Mullerian inhibiting substance and expression of the human gene in animal cells. *Cell* 1986;45:685–98.
- [2] Josso N, Picard JY. Anti-Mullerian hormone. *Physiol Rev* 1986; 66:1038–90.
- [3] Hirobe S, He WW, Lee MM, Donahoe PK. Mullerian inhibiting substance messenger ribonucleic acid expression in granulosa and Sertoli cells coincides with their mitotic activity. *Endocrinology* 1992;131:854–62.
- [4] Taketo T, Saeed J, Manganaro T, Takahashi M, Donahoe PK. Mullerian inhibiting substance production associated with loss of oocytes and testicular differentiation in the transplanted mouse XX gonadal primordium. *Biol Reprod* 1993;49:13–23.
- [5] Rey R, Lukas-Croisier C, Lasala C, Bedecarras P. AMH/MIS: what we know already about the gene, the protein and its regulation. *Mol Cell Endocrinol* 2003;211:21–31.
- [6] Durlinger AL, Kramer P, Karels B, de Jong FH, Uilenbroek JT, Grootegoed JA, Themmen AP. Control of primordial follicle recruitment by anti-Mullerian hormone in the mouse ovary. *Endocrinology* 1999;140:5789–96.
- [7] Baarends WM, Uilenbroek JT, Kramer P, Hoogerbrugge JW, van Leeuwen EC, Themmen AP, Grootegoed JA. Anti-mullerian hormone and anti-mullerian hormone type II receptor messenger ribonucleic acid expression in rat ovaries during postnatal development, the estrous cycle, and gonadotropin-induced follicle growth. *Endocrinology* 1995;136:4951–62.
- [8] Weenen C, Laven JS, Von Bergh AR, Cranfield M, Groome NP, Visser JA, Kramer P, Fauser BC, Themmen AP. Anti-Mullerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment. *Mol Hum Reprod* 2004;10:77–83.
- [9] Wunder DM, Bersinger NA, Yared M, Kretschmer R, Birkhauser MH. Statistically significant changes of antimullerian hormone and inhibin levels during the physiologic menstrual cycle in reproductive age women. *Fertil Steril* 2008;89: 927–33.
- [10] Sowers M, McConnell D, Gast K, Zheng H, Nan B, McCarthy JD, Randolph JF. Anti-Mullerian hormone and inhibin B variability during normal menstrual cycles. *Fertil Steril* 2010;94: 1482–86.
- [11] Tsepelidis S, Devreker F, Demeestere I, Flahaut A, Gervy C, Englert Y. Stable serum levels of anti-Mullerian hormone during the menstrual cycle: a prospective study in normo-ovulatory women. *Hum Reprod* 2007;22:1837–40.
- [12] La Marca A, Giulini S, Orvieto R, De Leo V, Volpe A. Anti-Mullerian hormone concentrations in maternal serum during pregnancy. *Hum Reprod* 2005;20:1569–72.
- [13] Freour T, Mirallie S, Bach-Ngohou K, Denis M, Barriere P, Masson D. Measurement of serum anti-Mullerian hormone by Beckman Coulter ELISA and DSL ELISA: comparison and relevance in assisted reproduction technology (ART). *Clin Chim Acta* 2007;375:162–4.
- [14] Al-Qahtani A, Muttukrishna S, Appasamy M, Johns J, Cranfield M, Visser JA, Themmen AP, Groome NP. Development of a sensitive enzyme immunoassay for anti-Mullerian hormone and the evaluation of potential clinical applications in males and females. *Clin Endocrinol (Oxf)* 2005;63:267–73.

- [15] Ball BA, Conley AJ, MacLaughlin DT, Grundy SA, Sabeur K, Liu IK. Expression of anti-Mullerian hormone (AMH) in equine granulosa-cell tumors and in normal equine ovaries. *Theriogenology* 2008;70:968–77.
- [16] Long WQ, Ranchin V, Pautier P, Belville C, Denizot P, Cailla H, Lhomme C, Picard JY, Bidart JM, Rey R. Detection of minimal levels of serum anti-Mullerian hormone during follow-up of patients with ovarian granulosa cell tumor by means of a highly sensitive enzyme-linked immunosorbent assay. *J Clin Endocrinol Metab* 2000;85:540–4.
- [17] La Marca A, Volpe A. The Anti-Mullerian hormone and ovarian cancer. *Hum Reprod Update* 2007;13:265–73.
- [18] Rey R, Sabourin JC, Venara M, Long WQ, Jaubert F, Zeller WP, Duvillard P, Chemes H, Bidart JM. Anti-Mullerian hormone is a specific marker of sertoli- and granulosa-cell origin in gonadal tumors. *Hum Pathol* 2000;31:1202–8.
- [19] McCue PM. Neoplasia of the female reproductive tract. *Vet Clin North Am Equine Pract* 1998;14:505–15.
- [20] Sundberg JP, Burnstein T, Page EH, Kirkham WW, Robinson FR. Neoplasms of Equidae. *J Am Vet Med Assoc* 1977;170:150–2.
- [21] MacLachlan NJ. Ovarian disorders in domestic animals. *Environ Health Perspect* 1987;73:27–33.
- [22] Neto AC, Ball BA, Browne P, Conley AJ. Cellular localization of androgen synthesis in equine granulosa-theca cell tumors: immunohistochemical expression of 17 α -hydroxylase/17,20-lyase cytochrome P450. *Theriogenology* 2010;74:393–401.
- [23] Gift LJ, Gaughan EM, Schoning P. Metastatic granulosa cell tumor in a mare. *J Am Vet Med Assoc* 1992;200:1525–6.
- [24] McCue PM, Roser JF, Munro CJ, Liu IK, Lasley BL. Granulosa cell tumors of the equine ovary. *Vet Clin North Am Equine Pract* 2006;22:799–817.
- [25] Roser JF, McCue PM, Hoye E. Inhibin activity in the mare and stallion. *Domest Anim Endocrinol* 1994;11:87–100.
- [26] Stabenfeldt GH, Hughes JP, Kennedy PC, Meagher DM, Neely DP. Clinical findings, pathological changes and endocrinological secretory patterns in mares with ovarian tumours. *J Reprod Fertil Suppl* 1979;27:277–85.
- [27] Munro C, Stabenfeldt G. Development of a microtitre plate enzyme immunoassay for the determination of progesterone. *J Endocrinol* 1984;101:41–9.
- [28] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25:402–8.
- [29] Yuan JS, Reed A, Chen F, Stewart CN Jr. Statistical analysis of real-time PCR data. *BMC Bioinformatics* 2006;7:85.
- [30] Cook CL, Siow Y, Taylor S, Fallat ME. Serum mullerian-inhibiting substance levels during normal menstrual cycles. *Fertil Steril* 2000;73:859–61.
- [31] Hehenkamp WJ, Looman CW, Themmen AP, de Jong FH, Te Velde ER, Broekmans FJ. Anti-Mullerian hormone levels in the spontaneous menstrual cycle do not show substantial fluctuation. *J Clin Endocrinol Metab* 2006;91:4057–63.
- [32] La Marca A, Malmusi S, Giulini S, Tamaro LF, Orvieto R, Levratti P, Volpe A. Anti-Mullerian hormone plasma levels in spontaneous menstrual cycle and during treatment with FSH to induce ovulation. *Hum Reprod* 2004;19:2738–41.
- [33] Jayaprakasan K, Deb S, Batcha M, Hopkisson J, Johnson I, Campbell B, Raine-Fenning N. The cohort of antral follicles measuring 2–6 mm reflects the quantitative status of ovarian reserve as assessed by serum levels of anti-Mullerian hormone and response to controlled ovarian stimulation. *Fertil Steril* 2010;94:1775–81.
- [34] Fanchin R, Schonauer LM, Righini C, Frydman N, Frydman R, Taieb J. Serum anti-Mullerian hormone dynamics during controlled ovarian hyperstimulation. *Hum Reprod* 2003;18:328–32.
- [35] Nelson SM, Stewart F, Fleming R, Freeman DJ. Longitudinal assessment of antimullerian hormone during pregnancy-relationship with maternal adiposity, insulin, and adiponectin. *Fertil Steril* 2010;93:1356–8.
- [36] Vigier B, Tran D, du Buisson F, Heyman Y, Josso N. Use of monoclonal antibody techniques to study the ontogeny of bovine anti-Mullerian hormone. *Reproduction* 1983;69:207–14.
- [37] Gustafson ML, Lee MM, Scully RE, Moncure AC, Hirakawa T, Goodman A, Muntz HG, Donahoe PK, MacLaughlin DT, Fuller AF. Mullerian inhibiting substance as a marker for ovarian sex-cord tumor. *N Engl J Med* 1992;326:466–71.
- [38] Rey RA, Lhomme C, Marcillac I, Lahlou N, Duvillard P, Josso N, Bidart JM. Anti-mullerian hormone as a serum marker of granulosa cell tumors of the ovary: comparative study with serum alpha-inhibin and estradiol. *Am J Obstet Gynecol* 1996;174:958–65.
- [39] Bailey MT, Troedsson MH, Wheato JE. Inhibin concentrations in mares with granulosa cell tumors. *Theriogenology* 2002;57:1885–95.
- [40] Zelli R, Sylla L, Monaci M, Stradaioi G, Sibley LE, Roser JF, Munro C, Liu IK. Gonadotropin secretion and pituitary responsiveness to GnRH in mares with granulosa-theca cell tumor. *Theriogenology* 2006;66:1210–18.