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Anti-Müllerian hormone profiling in prepubertal horses and its relationship with gonadal function

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ABSTRACT

Anti-Müllerian hormone (AMH) has gained increasing interest as a biomarker for assessment of gonadal activity. The ability to predict the ovarian follicular reserve of prepubertal female horses (fillies) or to identify stallions with testicular pathologies already during their prepubertal life has not been analyzed so far. Both would help to select fertile horses and reduce costs associated with keeping animals. The objectives of the present study were to (1) assess AMH, LH, FSH, progesterone (females) and testosterone (males) dynamics in prepubertal horses from birth onwards and (2) determine whether AMH concentrations detected in plasma of prepubertal female and male horses are correlated with postpubertal gonadal development. Warmblood foals (n = 30, 14 females, 10 normal males and 6 males with abnormal testicular development) born between February and May of two consecutive years (n = 28 in the first year and n = 2 the next year), were included in the study. Information on gestational length, parity of the dam and placental weight was collected for all foals. Blood samples for hormone analysis were collected from birth onwards every four weeks up to the age of one year. At two years, blood samples were collected on the day when antral follicle count (AFC) and total testicular volume (TTV) were assessed. AMH was detectable in the plasma of all animals from birth onwards and its concentration was significantly higher ($P < .001$) in males than in females, regardless of testicular development. In males, AMH and testosterone concentration were similar for all animals during the first year of life, regardless of testicular development. At two years, AMH concentration was higher ($P < .05$) in males with abnormal testicular development than in those with normal testes. In females, AMH concentration at two years was correlated with AMH concentration at birth ($P < .05$) and with AFC ($P < .001$). At birth, LH concentration was lower ($P < .05$) in stallions with abnormal testes (0.3 ± 0.2 ng/ml) than in controls (0.6 ± 0.2 ng/ml). A high negative correlation between AMH concentration and gestation length was observed in males during the first eight weeks of life ($P < .01$, $r = -0.64$ to -0.71). Elevated progesterone concentrations over 1 ng/ml were observed in several females starting with 20 weeks of age. This was paralleled by an increase in AMH concentration and was preceded by FSH and LH increases. In conclusion, AMH determination can be reliably used from two years onwards to identify stallions with abnormal testicular development, but it is inconclusive before puberty. In female horses, determination of AMH concentration at a prepubertal age allows for prediction of AMH and AFC after puberty. We suggest that premature luteinisation occurs before the onset of puberty in female horses and that LH secretion in the perinatal period is involved in testicular development and descent in the horse.

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1. Introduction

Selection of both male and female horses for breeding is usually performed at a mature age, i.e. not before two years in stallions and three years in mares. Development of a predictive method for determination of the ovarian follicular reserve in prepubertal female horses (fillies) and testicular development in prepubertal male horses (colts) would allow for selection of fertile horses for breeding at an early stage. However, at present such a method is not available for the horse.

The ovarian reserve of gonadotrophin-responsive follicles and embryo quality can be successfully predicted in women [1,2], cows [3], goats [4] and sheep [5] by determination of anti-Muellerian hormone (AMH) concentration in a single blood sample. In horses, besides its confirmed role in the diagnostic of granulosa-cell tumors [6], concentrations of AMH, a product of granulosa cells in pre-antral and small antral follicles, strongly correlates with antral follicle count (AFC) in older mares [7]. Moreover, a high long-term consistency of plasma AMH was observed within individuals, i.e. within individual mares, AMH concentration was not influenced by cycle stage or pregnancy [7]. Therefore, a wide variation of AMH concentration among individuals has been suggested to reflect the relative reproductive age of older mares [7], as described for women [8].

In male horses, AMH is expressed by Sertoli cells in fetal, neonatal and prepubertal testes, as well as in non-descended testes of cryptorchids [9]. At birth, serum AMH concentrations in neonatal colts are ten times higher than in neonatal fillies [10]. Secretion of AMH by the fetal testis not only induces regression of the paramesonephric duct, but also regulates testicular function in the prepubertal male by suppressing Leydig cell differentiation and steroidogenesis [11]. Sertoli cell maturation during puberty is accompanied by a decrease in the expression of AMH and its receptor [12] and a low circulating AMH in the normal stallion [9]. In the adult testis, AMH blocks the differentiation of mesenchymal cells into Leydig cells and decreases expression of steroidogenic enzymes [11,13]. Because AMH is a Sertoli cell-derived product, its concentration can be used to distinguish cryptorchidism from anorchia early in prepubertal boys [14], calves [15], but also in adult stallions [10]. Moreover, AMH concentrations are positively correlated with testicular volume and sperm concentration in men with maldescended testis, reflecting Sertoli cell number and function [16].

Puberty is a complex process during which individuals develop from a state of reproductive immaturity to a state of full reproductive competence. Onset of puberty in the female may be defined as the time of the first ovulation, which is associated with a subsequent increase in progesterone concentration reflective of a functional corpus luteum, nominally greater than 1 ng/ml [17]. Puberty in male animals has been defined as the time when the testes become active, and the first significant increase in testosterone concentration (greater than 2 SD above the baseline concentration for the assay) occurs [18]. According to semen characteristics (first ejaculate containing 50×10^6 spermatozoa with $\geq$10% motile), puberty occurred at 12–15 months in Welsh Pony colts [19] and at 14–24 months in Quarter Horse colts [20]. At present, there is no method by which future fertility or reproductive capacity can be predicted in males, though markers of Sertoli cell function, such as AMH have not been explored in stallions.

The objectives of the present study were to (1) assess AMH, luteinizing hormone (LH), follicle stimulating hormone (FSH), as well as progesterone (females) and testosterone (males) dynamics in prepubertal horses from birth onwards and (2) determine whether AMH concentrations detected in plasma of prepubertal female and male horses are correlated with postpubertal gonadal development.

2. Materials and methods

2.1. Animals

Warmblood foals (n = 30, 14 females and 16 males) born at the Brandenburg State Stud at Neustadt (Dosse), Germany, between February and May of two consecutive years (n = 28 in the first year and n = 2 the next year), were included into the study. Information on gestational length, parity of the dam and placental weight was collected for all foals included in this study. Until the middle of May, foals and their dams were kept in large group stables. Every day they spent several hours in outdoor paddocks. Thereafter, the foals were kept on pasture all day and in a spacious group stable at night. Foals were weaned in October (age: 27 weeks ± 5 days). After weaning they were grouped by sex and again kept in large group stables with daily access to outdoor paddocks. Horses were fed oats and minerals twice daily and hay ad libitum. Experiments were performed in agreement with German animal welfare legislation and approved by the Brandenburg State Ministry for Rural Development, Environment and Consumer Protection (licence number: 2347-A-5-1-2014 A1).

2.2. Hormone assays

Blood samples were collected from the jugular vein into heparinized tubes from birth onwards every four weeks until 52 weeks of age. At two years of age, blood samples were collected on the day of gonadal assessment (see 2.3 and 2.4). After centrifugation at 1200×*g* for 10 min plasma was collected and stored at −20 °C until further analysis.

Plasma AMH concentrations were determined with an enzyme-linked immunosorbent assay as recommended by the manufacturer (AL-115, Ansh Labs, Webster, TX, USA). Serial dilutions of equine plasma showed good parallelism to the AMH standard curve (deviation within 12% of expected values). The mean recovery of the standard added to equine plasma before assaying was 77%. The intra- and inter-assay coefficients of variation were 4.6% and 7.9%, respectively, and the minimal detectable concentration was 0.07 ng/ml.

Progesterone concentrations were determined with an enzyme-linked immunosorbent assay (ADI-901-011, Enzo Life Sciences, Farmingdale, NY, USA) as described [21]. Serial dilutions of equine plasma showed good parallelism to the standard curve (deviation within 20% of expected values). The mean recovery of the standard added to equine plasma samples before assaying was 96%. Plasma was diluted 1:100 before analysis. The intra- and inter-assay coefficients of variation were 5.3% and 5.5%, respectively, and the minimal detectable concentration 0.08 ng/ml.

Testosterone concentrations were determined with an enzyme-linked immunosorbent assay (DE1559, Demeditec, Kiel-Wellsee, Germany) as described [22]. Serial dilutions of equine plasma showed good parallelism to the testosterone standard curve (deviation within 17.5% of expected values). The mean recovery of the standard added to equine plasma samples before assaying was 107.4%. Plasma was not diluted before analysis. The intra- and inter-assay coefficients of variation were 9.4% and 8.4%, respectively, and the minimal detectable concentration was 0.01 ng/ml.

Concentrations of FSH were determined using a previously validated RIA [23], with slight modifications. Highly purified pituitary derived equine FSH (e265B, Papkoff, UC Davis, CA) was used for standards (ranging from 0.25 to 64 ng/mL) and for iodination. The primary antibody was LSU 3D-2 anti-oFSH from Dr. D.L. Thompson, LSU, Baton Rouge LA, used at a 1:20,000 dilution. One

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